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The Isolation, Characterisation and Culture of Putative Human Liver Progenitor Cells

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Abstract

The aim of this thesis was to investigate diseased human livers for the presence of liver progenitors. There is a shortage of organ donors and the difficulty in treating acute and chronic liver diseases puts emphasis on the need for alternative therapies to orthotopic liver transplants.

The long term aim is to find a cell source which could populate a bio-artificial liver device. Liver stem cells would have the capacity to expand into large enough numbers needed for such a device and to differentiate into functional liver cells.

Non-parenchymal cells were isolated from human liver, notably from livers explanted from patients with acute liver failure or cirrhosis. The underlying hypothesis was that livers removed at transplantation would be enriched in hepatocyte progenitors as they are trying to regenerate and repair the damaged tissue. Putative liver progenitor colonies were identified and characterised by (a) culture of non-parenchymal cells and observation for adoption of a mature hepatocyte phenotype and (b) assayed for the presence of stem cell and mature liver cell lineage markers (hepatocytes and biliary epithelial cells). Markers were investigated using RT-PCR, immunocytochemistry (on cytospins and *in-situ* in culture wells) and FACS analysis. Markers studied included c-met (HGF-receptor), CD49f (α6-integrin), haematopoietic stem cell markers CD117 and CD133, and multidrug resistance protein ABCG2/BCRP. Proliferating colonies were also manipulated by immortalisation (hTERT) and cultured with either growth factors thought to be likely to induce hepatocyte proliferation and/or factors associated in differentiation.

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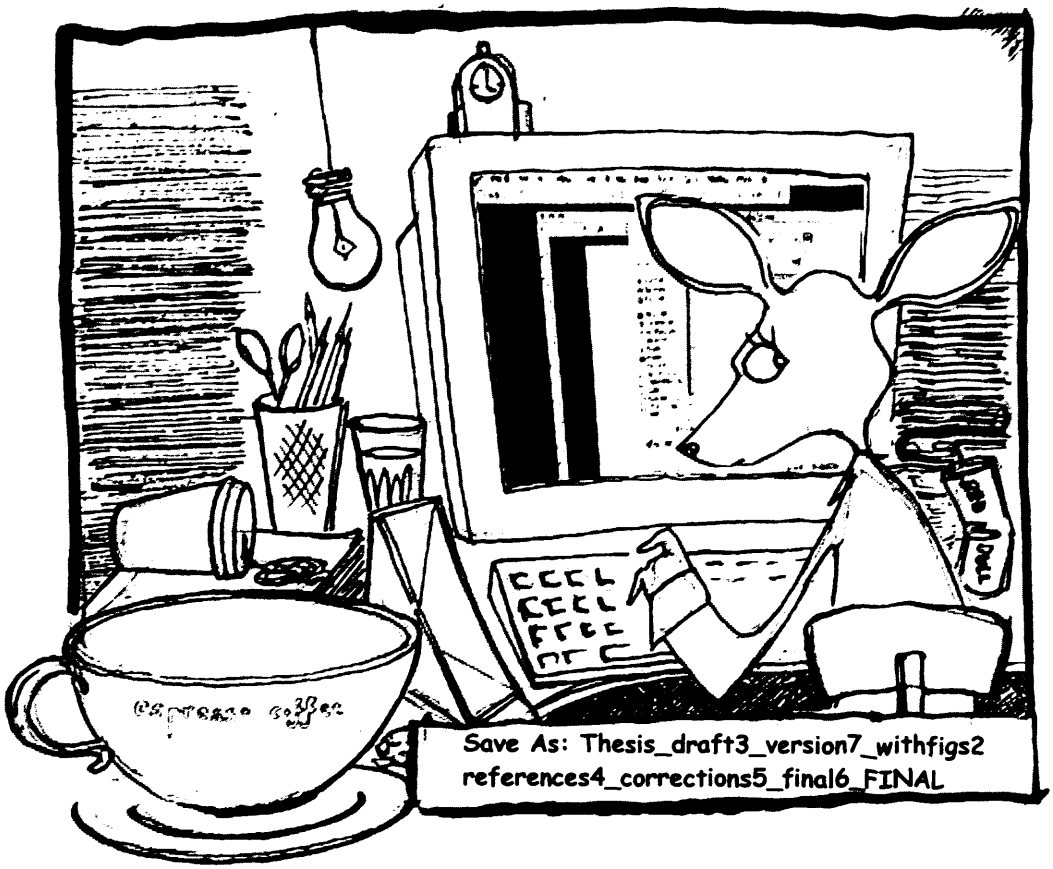
Cartoon by Åsa Lucander



Abbreviations

AAT	Alpha-1-Antitrypsin
AAF	2-acetylaminofluorene
Ab	Antibody
ABCG2	ATP-binding cassette subfamily G member 2
aFGF	acidic Fibroblast Growth Factor
AFP	Alpha Fetoprotein
Alb	Albumin
ALD	Alcohol Liver Disease
ALDH	Aldehyde Dehydrogenase
ALT	Alternative Lengthening of Telomeres
APC	Allophycocyanin
ATP	Adenosine triphosphate
bFGF	basic Fibroblast Growth Factor
BGP	Biliary Glycoprotein
BMP	Bone Morphogenic Protein
BS	Normal liver resection
BSA	Bovine Serum Albumin
CCl_4	Carbon tetrachloride
CFC	Colony Forming Cell
CK	Cytokeratin
Cry	Cryptogenic
C/EBP	CAAT Enhancer Binding Protein
DAB	3,3'-diaminobenzidine chromogen solution
DDC	Diethyldithiocarbamic acid
DIG	Digoxigenin
DLK	Delta-like
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulphoxide
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGTA	Ethylene Glycol Bis-2-Aminoethyl Ether-N,N',N",n'-Tetraacetic Acid
EDTA	Ethylenediaminetetra-acetic acid

ELISA	Enzyme Linked Immunoabsorbent Assay
ES	Embryonic Stem
FAH	Fumarylacetoacetate hydrolase
FCM	Flow cytometry
FCMCS	Flow cytometric cell sorting
FCS	Foetal Calf Serum
FBS	Foetal Bovine Serum
FFPE	Formalin Fixed Paraffin Embedded
FGF	Fibroblast Growth Factor
FITC	Fluorescein Isothiocyanate
FSC	Forward Scatter
FTC	Fumitremorgin C
Ful	Fulminant
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gata	GATA binding transcription factor
GCSF	Granulocyte-Colony-Stimulating Factor
GF	Growth Factor
GFP	Green Fluorescent Protein
GGT	Gamma Glutamyl Transpeptidase
GM-CSF	Granulocyte-Macrophage-Colony-Stimulating Factor
GST	Glutathione-S-transferase
G6P	Glucose-6 phosphatase
HBSS	Hanks Buffered Salts Solution
H-CFU-C	Hepatic Colony-Forming Units in Culture
HEA125	Human Epithelial Antigen 125
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HGF	Hepatocyte Growth Factor
HNF	Hepatocyte Nuclear Factor
HRP	Horseradish Peroxidase
HSA	Human Serum Albumin
HSC	Haematopoietic Stem Cell
H&E	Haematoxylin and Eosin
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IL	Interleukin

LDL	Low-Density Lipoprotein
LIF	Leukaemia Growth Factor
Lin	Lineage
MACS	Magnetic Cell Sorting
MAPC	Multipotent Adult Progenitor Cell
MEF	Mouse Embryonic Fibroblast
MCSF	Macrophage-Colony-Stimulating Factor
MDR	Multi-Drug Resistance
MGDF	Megakaryocyte Growth and Development Factor
MIP	Macrophage Inflammatory Protein
MPC	Mesenchymal Progenitor Cell
MSC	Mesenchymal Stem Cell
NGF	Nerve Growth Factor
npc	Non-parenchymal cells
OSM	Oncostatin M
OC	Oval Cell antigen
PAI	Plasminogen Activator Inhibitor
PBC	Primary Biliary Cirrhosis
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Monocyte
PDGF	Platelet-Derived Growth Factor
PE	Phycoerythrin
PF	Platelet Factor
Pgp	P-glycoprotein
PH	Partial Hepatectomy
PM	Proliferating Media
PSC	Primary Sclerosing Cholangitis
R	Region
RA	Retinoic acid
RNase	Ribonuclease
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SAGE	Serial Analysis of Gene Expression
SA-β-gal	Senescence-associated β-galactosidase
Sca	Stem cell antigen
SCF	Stem Cell Factor

SD	Standard Deviation
SDF	Stromal cell Derived Factor
SP	Side Population
SSEA	Stage-Specific Embryonic Antigen
SSC	Side Scatter
Stat	Signal Transducer and Activator of Transcription
Sub-ful	Sub-fulminant
TAE	Tris Acetic Acid EDTA
TAT	Tyrosine Aminotransferase
TBS	Tris-Buffered Saline
TGF	Transforming Growth Factor
TMB	3,3',5,5'-tetramethylbenzidine
TNF	Tumour Necrosis Factor
TO	Tryptophan-2, 3-dioxygenase
TRAP	Telomeric Repeat Amplification Protocol
TTR	transthyretin
TWEAK	TNF-like Weak inducer of apoptosis
UCB	Umbilical Cord Blood
VCAM	Vascular Adhesion Molecule
VEGRF	Vascular Endothelial Growth Factor
2AAF	2-acetylaminofluorene
3D	Three-Dimensional
α-MEM	alpha-Minimum Essential Medium
α-SMA	alpha-Smooth Muscle Actin

Chapter 1 - Introduction

The work was carried out at the Centre for Hepatology, Department of Medicine at the Royal Free Hospital. The aim of this thesis was to utilise liver cell biology to investigate liver stem cell potential for therapeutic use in liver disease.

1.1 Why liver stem cells?

Millions of people are affected by liver failure world-wide (up to 1 in 10 people in America) and more than 3000 people die each year in the UK as a result of liver disease. Liver failure can result from any type of liver disorder, including hepatitis, cirrhosis and liver damage from alcohol or drugs. The diseases can develop rapidly over days or weeks (acute liver failure) or gradually over months or years (chronic liver failure). People receiving a liver transplant have a high survival rate (around 80%), but there is a shortage of donor livers. Moreover, the progression of acute liver failure is often so rapid, that no suitable organs can be obtained in the available timeframe. New alternative or temporary therapies are being sought.

Some therapeutic advances have been made using artificial liver devices where toxins can be removed with sophisticated filters. These devices can help maintain minimal detoxifying liver functions until a suitable transplant has been found, or help tilt the balance between disease and recovery in the right direction. However, detoxification is only one of the important functions that the liver performs. The normal processes of the liver include storage (vitamins, cholesterol), synthesis (clotting factors, bile acids), regulation (glucose, fat, hormones), filtration (nutrients, poisons from gut, bile acids) and removal (bilirubin, ammonia, drugs, poisons) of important compounds. To restore the complex functions of the normal liver, a liver support device should contain a biological component. To replace the complex functions of the liver, a very large number of exceptionally well functioning cells are required. A rough estimate of the required cells to replace 100% of liver function is around $2x10^{11}$ (Selden and Hodgson, 2004).

Different sources of cells could be used for a bioartificial liver. Cell lines have great proliferative capacity, but lack a full repertoire of normal hepatocyte functions, and those functions expressed are often at lower levels than found *in vivo*. Experiments in our laboratory have found that some cell functions are improved in the HepG2 cell line when cultured in a 3D-system of alginate beads instead of a monolayer. Increased protein synthesis and secretion (albumin [alb], prothrombin, fibrinogen, alpha-1-antitrypsin [AAT] and alpha-1-acid glycoprotein) and steroid metabolism and cytochrome P450 enzyme function has been observed (Selden and Hodgson, 2004). However, the cells lack certain important functions, such as urea synthesis, and the cell lines used are often tumour derived, introducing safety issues.

Another alternative cell source for a bioartificial liver device is primary hepatocytes isolated from human liver. Hepatocytes have also been used for cell transplantation experiments with varying success mainly due to low engraftment (reviewed in Selden and Hodgson, 2004). *In vitro* studies have shown that primary cells are functional for short periods in culture, but their use is limited by low proliferation rates. Furthermore, primary hepatocytes of good quality are difficult to obtain as livers are normally used for transplantation and therefore the livers available for hepatocyte isolation are often of poor quality (Selden and Hodgson, 2004; Tosh and Strain, 2005).

However, the potential of liver stem cells in both a bioartificial liver and cell transplantation context is extensive. Stem cells have great proliferative capacity (similar to cell lines), so large numbers of cells could be derived from a very small isolated fraction of the liver. These cells could then be induced to differentiate and gain nearly perfect function (similar to primary hepatocytes). In addition, if the stem cells can be isolated from the patient (autologous) they would be genetically identical to the person and rejection or other complications due to immune reactions towards the graft could be avoided.

Furthermore, stem cells are ideal targets for gene therapy. Theoretically only one cell would need to be genetically manipulated, which could then be expanded into large numbers of cells with the correct genetic information.

In conclusion, liver stem cells are promising candidates for liver therapy. The cells could be utilised as a component of a bio-artificial liver device or for cell transplantation to aid recovery of the damaged liver by providing a source of functional liver cells. Most importantly of all, if natural liver regeneration in the body were better understood, the information could be used to treat patients in order to successfully activate natural recovery.

1.2 Where should liver stem cells be isolated from?

To understand how liver stem cells can be derived, an introduction to stem cell biology is necessary. Stem cells have the capability to self-renew and to produce daughter cells that are able to differentiate into cell types of different lineages. Theoretically, using these cells it would be possible to produce any cell type, tissue or organ to treat a patient.

There are different categories of stem cells. These range in differentiation potential from pluripotent cells, e.g. embryonic stem (ES) cells capable of differentiating into any cell of the body, to bipotential stem cells, e.g. oval cells of the liver, capable of differentiating along the hepatocyte or biliary epithelial route.

Probably the most well known example of stem cells is the multipotent haematopoietic stem cell (HSC). These progenitors are capable of differentiating into any lymphoid or myeloid cell involved in the blood, bone marrow, spleen or thymus. These cells have an enormous proliferative capacity and it has been shown in murine models that a single stem cell can regenerate and maintain the entire lymphohaematopoietic system (Szilvassy, 2003).

A similar system is thought to be present for most major adult organs and tissues, as stem cells also play an important role in tissue homeostasis by repair and maintenance (Blau et al., 2001). However, stem cells are rare, from around 0.0001% to 5% of the total cells in a tissue, and their identification and isolation has only been achieved in very few cases (Reya et al., 2001; Robert, 2004). At present adult stem cells have been identified from blood, brain, muscle, gut, skin, pancreas and liver (Bunting and Hawley, 2003).

The differentiation potential of adult stem cells was first thought to be relatively restricted. However, recent evidence suggests that they might be more flexible than expected (Vogel, 1999; Fuchs and Segre, 2000; Blau et al., 2001). Some adult stem cells (mainly haematopoietic stem cells) are thought to retain the ability to trans-differentiate (or trans-determinate), i.e. to differentiate into a cell type of another organ. An attractive hypothesis suggested by Blau *et al.* is that stem cells are not a discrete cellular entity but a biological function that can be induced in many distinct types of cells (Blau et al., 2001). However, it still remains controversial as to how important such cross-boundary events are under normal circumstances. Adult stem cell plasticity is discussed in more detail later in this chapter.

Because of the nature of stem cell plasticity, liver progenitors could be derived from ES cells, foetal liver cells, cord blood, adult liver or even from other adult stem cell types such as HSCs and mesenchymal stem cells [Fig. 1-1]. Even though ES cells, isolated from embryonic tissue, are thought to be the most flexible and promising set of stem cells, there are ethical issues involving the acquisition and use of this material. More committed cells derived from foetal tissues, such as foetal liver cells, have similar concerns. Adult stem cells bypass these problems and they hold the promise of being isolated from the patients own body. The use of animal material is limited due to safety concerns associated with cross-species therapy, e.g. zoonotic infections and ethics.

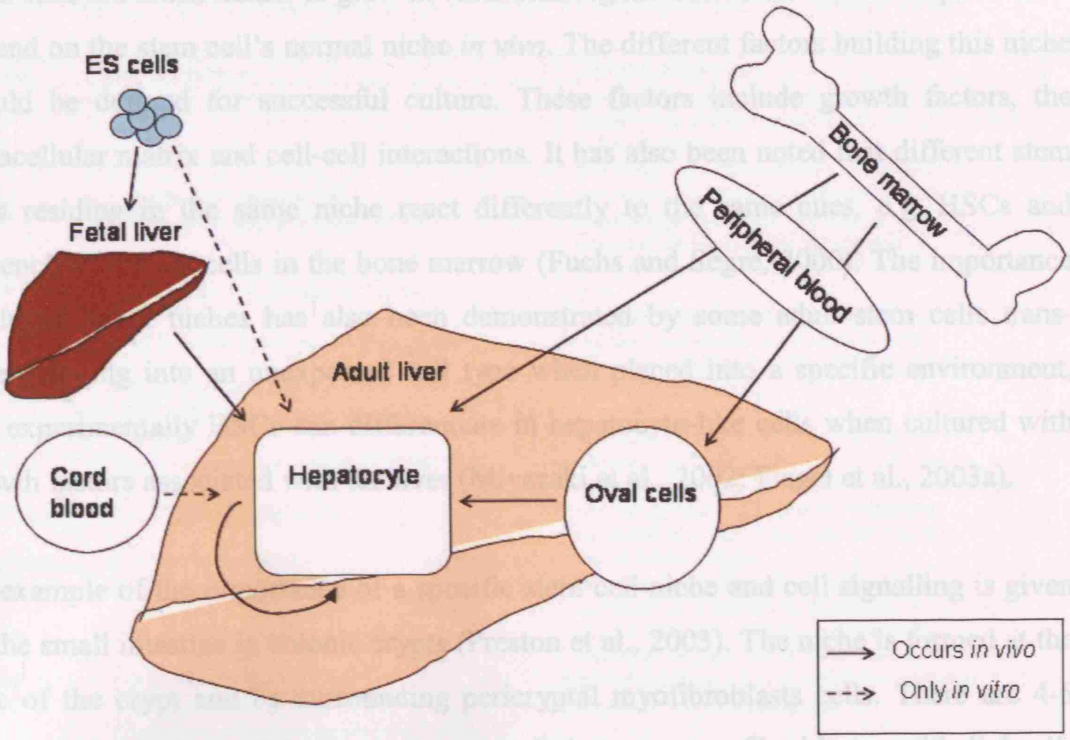


Fig. 1-1 A schematic representation of various sources of hepatocytes both in vivo and in vitro.

Diseased adult livers are thought to be enriched in liver stem cells and hepatocyte progenitors because the liver is trying to regenerate. The use of extrahepatic sources of adult stem cells for liver therapy is still controversial because of the ongoing debate over the amount and the type of contribution, i.e. trans-differentiation versus cell fusion, of these cells towards liver generation. These questions will be discussed in more detail later in this chapter.

1.3 What is required for the use of liver stem cells therapeutically?

The first task is to clearly define liver stem cells. At present there are no definite markers to identify a liver stem cell although many have been discussed (Tosh and Strain, 2005). Once the progenitors are isolated, the cells have to be kept in culture first to proliferate extensively without differentiation and then to differentiate into the required cell type.

Stem cells are much harder to grow *in vitro* than regular cell lines. Culture requirements depend on the stem cell's normal niche *in vivo*. The different factors building this niche should be defined for successful culture. These factors include growth factors, the extracellular matrix and cell-cell interactions. It has also been noted that different stem cells residing in the same niche react differently to the same cues, e.g. HSCs and mesenchymal stem cells in the bone marrow (Fuchs and Segre, 2000). The importance of the different niches has also been demonstrated by some adult stem cells trans-differentiating into an unexpected cell type when placed into a specific environment, e.g. experimentally HSCs can differentiate in hepatocyte-like cells when cultured with growth factors associated with the liver (Miyazaki et al., 2002; Fiegel et al., 2003a).

An example of the importance of a specific stem cell niche and cell signalling is given by the small intestine in colonic crypts (Preston et al., 2003). The niche is formed at the base of the crypt and by surrounding pericryptal myofibroblasts cells. There are 4-5 stem cells in the base of the crypt. A cross-talk between myofibroblasts, epithelial cells and stromal cells (expressing keratinocyte growth factor and hepatocyte growth factor [HGF]) is needed. It has also been shown that mice lacking a certain functional allele (high mobility group box transcription factor Tcf-4) cannot maintain the proliferative competence of the small intestine crypts (Preston et al., 2003).

As liver stem cells are not clearly defined, the isolation, characterisation, culture and differentiation should involve the consideration of related research from animal models, other stem cell areas as well as liver development and disease. It is important to note that although various animal models exist, it has been shown in many instances that stem cell biology differs largely between species.

In this chapter the different sources of liver stem cells are discussed in order to understand liver progenitor biology, as well as generate ideas of possible liver stem cell markers (see also Table 1-1 and Table 1-2) and culture conditions both to maintain stem cells and to differentiate the cells to mature liver cells. Section 1.4 introduces embryonic stem cells, 1.5 foetal liver cells and 1.6 stem cells from umbilical cord blood and placenta. 1.7 aims to explain what is known about adult liver stem cells, both in animal models and human disease. Other sources of liver stem cells are discussed in 1.8 from adult haematopoietic stem cells [1.8.1] and mesenchymal stem cells [1.8.2].

Table 1-1 List of surface markers used in order to characterise progenitor cells.

Marker	Also known as	Function/Expression
C1rRp	complement protein receptor	involved in classical complement pathway
CD7	gp40	expressed on pluripotent hematopoietic cells, T-cells and thymocytes
CD13	gp150, aminopeptidase N	expressed on pluripotent granulocytes and monocytes, bile ducts and bone marrow stroma
CD14	lipopolysaccharide receptor (LPS-R)	expressed on monocytes and macrophages
CD29	GP11a, VLA-β, β1 integrin sububit	expressed on hepatoblasts and maintained in maturation of both hepatocyte and biliary epithelial cells
CD34	GP105-120, ligand for CD62 (L-selectin)	expressed on pluripotent hematopoietic cells, capillary endothelium and embryonic liver
CD38	T-10, ADP-ribosylcyclase	augments B-cell proliferation and expressed on lymphoid progenitors
CD44	H-CAM, Pgp-1, Hermes ag, ECMR II, HUTCH-1, gp85	expressed on leukocytes and erythrocytes
CD45	leukocyte common ag (LCA), B220, T200	expressed on leukocytes
CD49alpha	VLA-1α, α1 integrin	expressed on hepatoblasts, maintained in hepatocyte and lost in biliary epithelial cell maturation
CD49beta	VLA-2α, α2 integrin	expressed on mature biliary epithelial cells, associates with CD29
CD49c	VLA-3α, α3 integrin	expressed on mature biliary epithelial cells
CD49e	VLA-4α, α4 integrin	expressed on hepatoblasts, maintained in hepatocyte and lost in biliary epithelial cell maturation
CD49f	VLA-6α, α6 integrin	expressed on hepatoblasts, maintained in biliary epithelial cells and lost in hepatocyte maturation
CD71	T9, transferrin receptor	expressed on proliferating cells
CD106	vascular adhesion molecule-1 (VCAM-1), INCAM110	Ligand for VLA-4, expressed on endothelial cells
CD117	c-kit, stem cell factor receptor (SCFR)	expressed on hematopoietic progenitors, mast cells and liver stem cells
CD123	interleukin-3 receptor (Il-3Rα)	expressed on bone marrow stem cells, granulocytes, monocytes and megakaryocytes
CD124	interleukin-4 receptor (Il-4Rα)	expressed on hematopoietic precursors and mature B - and T-cells
CD133	AC133, PROML1, hematopoietic stem cell ag	expressed on hematopoietic, brain and breast stem cells
c-met	hepatocyte growth factor receptor (HGFR)	receptor for HGF which is a mitogen, motogen and morphogen
CXCR-4	stromal cell derived factor 1 (SDF-1R)	role in attracting stem cells
Flk1	vascular endothelial growth factor receptor 2 (VEGFR2), KDR	expressed on early hematopoietic progenitor cells
Flt1	vascular endothelial growth factor receptor 1 (VEGF1)	expressed on early hematopoietic progenitor cells and monocytes and megakaryocyte precursors
lin	lineage marker	a combination of markers of haematological or lymphoid differentiation
MDR1, MRP1, MRP3	ATP-binding cassette transporter proteins	ABC transporter proteins
OC.2, OV-6, BD1, A6, SH2/3		antibodies raised aginst rodent and human oval cells and biliary epithelial cells
OX43		expressed on macrophages, endothelial cells and red cell precursors
OX44	CD53	expressed on myeloid and peripheral lymphoid cells
Sca-1	stem cell antigen 1, lymphocyte activation protein 6A (Ly-6A)	expressed on hematopoietic progenitor cells and oval cells
SSEA-1	stage specific embryonic antigen-1	expressed on undifferentiated rodent ES cells and differentiated human ES cells
SSEA-3	stage specific embryonic antigen-3	expressed on undifferentiated human ES cells and differentiated rodent ES cells
SSEA-4	stage specific embryonic antigen-4	expressed on undifferentiated human ES cells and differentiated rodent ES cells
TER119		erythroid precursor marker
Thy-1	CD90	expressed on hematopoietic progenitors and liver progenitors (oval cells)
Tra-1-61	keratan sulphate-associated antigen	expressed on undifferentiated ES cells
Tra-1-80	keratan sulphate-associated antigen	expressed on undifferentiated ES cells

1.4 Embryonic stem cells

Embryonic stem (ES) cells are pluripotent, i.e. the cells can form tissues of all three germ layers but are unable to form an entire living being. The cells are derived from the inner cell mass of the blastocyst (Schatten et al., 2005). In this section the markers and normal culture requirements of ES cells are discussed, whereafter the differentiation potential into hepatocytes is presented.

1.4.1 ES cell markers

ES cell biology is very complex, hence the markers and their functions are only introduced briefly in this section. Some of the markers are analysed further in the later chapters to discuss their importance with respect to the results presented in this thesis.

Carpenter *et al.* compared 26 human ES cell lines to identify the common characteristics of ES cells (Carpenter et al., 2003). The cells all retained normal karyotype, showed similar expression patterns of surface markers Stage-Specific Embryonic Antigen (SSEA)-3, SSEA-4, keratan sulphate-associated antigens Tra-1-60 and Tra-1-81 (Henderson et al., 2002), as well as, markers associated with pluripotency (e.g. Oct-4). The cells had high levels of telomerase activity and hTERT expression and appeared to be immortal (have indefinite growth potential). The cells could also differentiate into derivatives of all three germ layers. Furthermore, Carpenter *et al.* observed CD133 expression in all cell lines. However, the marker was only expressed by 50-60% of the cells, compared to 70-100% expression of SSEA-4, Tra-1-60 and Tra-1-81. Undifferentiated ES were also found to be tumorgenic in the experimental set-up.

The most interesting markers on undifferentiated ES cells are the surface markers SSEA-1, SSEA-3 and SSEA-4 and Tra-1-60 and Tra-1-81 (Henderson et al., 2002). An example of interspecies differences is demonstrated with these markers where undifferentiated mouse ES cells express SSEA-1 and increase expression of SSEA-3 and SSEA-4 when differentiating. However, the opposite is seen with human ES cells. Undifferentiated human ES cells express SSEA-3, SSEA-4, Tra-1-60 and Tra-1-81. Differentiation is characterised by the increased expression of SSEA-1 and the downregulation of SSEA-3 and SSEA-4 (Shamblott et al., 1998; Thomson et al., 1998; Reubinoff et al., 2000; Henderson et al., 2002; Draper et al., 2002). The SSEA-1 has

been identified to be identical to Lewis X and CD15, which is a blood group antigen involved in adhesion. The specific functions of the other markers are unknown (Henderson et al., 2002) .

ES cells also express the transcription factor Oct-4, a POU domain transcription factor. In ES cells the expression of Oct-4 is down regulated with differentiation. (Reubinoff et al., 2000). Oct-4 is essential for development of pluripotent cells in the mouse embryo, since Oct-4 deficient embryos develop to the blastocyst stage, but the inner cell mass cells are not pluripotent. Oct-4 expression in mice is restricted *in vitro* to undifferentiated ES cells. *In vivo*, however, Oct-4 is also expressed in the germline throughout development and has also been found to be elevated in some tumours (Nichols et al., 1998).

It has been shown that human ES cells express low levels of different growth factor receptors (Schuldiner et al., 2000). These included the receptors for the following growth factors: basic fibroblast growth factor (bFGF), transforming growth factor β1 (TGF-β1), activin-A, bone morphogenic protein 4 (BMP-4), hepatocyte growth factor (HGF), epidermal growth factor (EGF), β nerve growth factor (βNGF) and retinoic acid. When the growth factors were added to the culture media, none of them exclusively directed differentiation to a particular cell type but altered the relative proportions of cell types in the culture. The growth factors associated with liver growth and development are HGF and EGF. The addition of EGF activated ectodermal and mesodermal markers (but not endoderm), but HGF allowed differentiation into the three embryonic germ layers (Schuldiner et al., 2000).

Another interesting marker, Nanog, was identified by Mitsui *et al.* and Chambers *et al.* in mouse (Mitsui et al., 2003; Chambers et al., 2003). Nanog is capable of maintaining ES cell renewal and pluripotency independently of the leukaemia inhibitory factor [LIF]/ receptor gp130/ Signal Transducer and Activator of Transcription3 [Stat3] pathway. Nanog might maintain self-renewal by transcriptional repression of genes that promote differentiation, such as *gata4* (GATA binding transcription factor) and *gata6*. An important link between Oct-4 and Nanog was also identified. Nanog was found to be expressed in pluripotent human cell lines but not in differentiated cells.

1.4.2 Culture of undifferentiated embryonic stem cells

Keeping ES cells undifferentiated has been one of the biggest challenges of ES cell biology because the cells differentiate spontaneously if the pluripotent state is not actively maintained. Thomson *et al.* investigated the maintenance of human ES cells (Thomson et al., 1998). It was found that the cells would differentiate without a mouse embryonic fibroblast (MEF) feeder layer. Furthermore, the cells would differentiate even if grown on feeders, if grown to confluency. Leukaemia Inhibitory Factor (LIF), which allows mouse ES cells to be grown without feeders, was not sufficient to maintain pluripotency, again demonstrating the difference between mouse and human ES cells. Human ES cells also need to be grown in colonies > 50-100 cells, as a single cell will perish (Thomson et al., 1998).

Human ES cells have been grown without feeder cell layers. Xu *et al.* showed this by using matrigel or laminin (extracellular matrix proteins) and MEF-conditioned media (Xu et al., 2001). The ES cells cultured in this way retained the characteristics of human ES cells. Conditioned media alone was not enough as there was a requirement for undefined soluble factors in conjunction with the extracellular matrix. Richards *et al.* have developed a human feeder approach, which removes the need for animal material for human ES cell culture (Richards et al., 2002).

1.4.3 Embryonic Stem cells differentiate into hepatocytes

Cells expressing hepatocyte markers and function have been produced from ES cells isolated from rats, mice and humans using different differentiation methods.

For rodents, differentiation to hepatocytes has been observed from rat ES cells using a collagen gel system (supplemented with LIF) (Tabei et al., 2003) and from mouse ES cell derived embryoid bodies (15 days after removal of LIF) (Chinzei et al., 2002). The isolated hepatocyte cells showed expression of either albumin and bilirubin (Tabei et al., 2003) or alpha-fetoprotein (AFP), albumin and functional urea synthesis (Chinzei et al., 2002).

Hamazaki *et al.* were able to differentiate mouse ES cells without additional growth factors to express AFP, transthyretin [TTR], alpha-1-antitrypsin [AAT] and albumin. Further differentiation was induced by growth factors (acidic fibroblast growth factor

[aFGF], HGF and Oncostatin M [OSM]) and resulted in expression of late differential markers, e.g. tyrosine aminotransferase, glucose-6 phosphatase [G6P]) (Hamazaki et al., 2001).

HGF and β-nerve growth factor were also found to induce differentiation of mouse ES cells into hepatocyte-like cells (expressing AAT, AFP, albumin, TTR, G6P and hepatic nuclear factor 4 [HNF4]) after 15 days of culture (Kuai et al., 2003). Transfection of mouse ES cells with HNF3β, grown in a three-dimensional culture system with FGF-2 had a similar effect. The differentiated cells produced albumin, triacylglycerol and could synthesise urea (Ishizaka et al., 2002).

Yamamoto *et al.* used a different approach by transplanting mouse ES cells transduced with green fluorescent protein (GFP) under the albumin promoter, into injured livers of carbon tetrachloride (CCl_4) treated mice (Yamamoto et al., 2003). Improved liver function was observed in the mice and the GFP expressing cells (i.e. albumin expressing ES derived cells) were analysed and isolated from the livers of the recipient mice. Around $2x10^6$ ES cells differentiated into approximately $3x10^7$ cells expressing liver markers (Yamamoto et al., 2003).

For human ES cell lines differentiation into hepatocyte-like cells using dimethyl sulfoxide (DMSO) and sodium butyrate has been reported (Rambhatla et al., 2003). The differentiated cells gained hepatocyte morphology, expressed albumin, AAT, cytokeratin 8 (CK8) and 18, accumulated glycogen and had inducible cytochrome P450 activity (Rambhatla et al., 2003).

Interestingly, Yin *et al.* isolated 'tissue specific' stem cells from differentiating ES cells by using the foetal liver marker AFP. ES cells were transduced with GFP under an AFP promoter. Cells expressing AFP therefore expressed GFP and could be isolated and transplanted to 2/3 partially hepatectomized mice. The cells were found to differentiate into albumin-positive hepatocytes (Yin et al., 2002b).

Even though ES cells, or hepatic sub-sets of ES cells, have been found to be able to differentiate into hepatocyte-like cells in murine models, the efficiency of differentiation (percentage of cells expressing hepatocyte markers) is variable and not

always reported. For example Pan *et al.* investigated the differentiation ratio of mouse ES cells and found that there was only 5.5% differentiated cells on day 13 and 10.4% on day 21 after LIF removal (Pan et al., 2005).

Importantly, there is also a risk of malignant transformations or uncontrolled proliferation due to the tumorigenic properties of undifferentiated ES cells. The safety concerns as well as the ethical issues for the study and potential use of ES cells makes the use of these cells clinically difficult (Dahlke et al., 2004; Dahlke and Schlitt, 2003; McLaren, 2001).

1.5 Foetal liver cells

Foetal liver cells and hepatoblasts are the developmental precursors or stem cells of the liver, able to differentiate into both hepatocytes and biliary epithelial cells. The cells often induce less graft versus host disease after transplantation and are more flexible to work with than adult cells, but again raise both safety and ethical issues regarding their isolation and use. This section introduces different studies of foetal liver cells isolated from rodents and humans.

Different integrins are expressed during human embryogenesis (Couvelard et al., 1998). Hepatoblasts have been found to express β1, α1, α5, α6 and α9 integrin chains (including the α1β1, α5β1, α6β1 [CD49f CD29] and α9β1 dimers). The level of these integrins decrease in foetal hepatocytes.

The isolation of hepatoblasts/foetal liver stem cells and their subsequent culture and/or differentiation has been shown in several studies. Nitou *et al.* isolated mouse hepatoblasts using E-cadherin as a marker (Nitou et al., 2002). The cells expressed AFP and albumin but lacked other markers of mature hepatocytes. The co-culture of the hepatoblasts together with non-parenchymal cells was found to be essential for their mutual survival, proliferation, differentiation and morphogenesis.

To differentiate between haematopoietic and hepatic progenitors in foetal liver, Fiegel *et al.* used depletion of cells expressing OX43 (macrophages, endothelial cells and red-cell

precursors) and OX44 (myeloid and peripheral lymphoid cells) and found that hepatic progenitors in rat expressed Thy1 [CD90] (Fiegel et al., 2003b).

Bipotential cell lines have also been isolated and characterised from mouse embryos by Strick-Marchand *et al.* (Strick-Marchand and Weiss, 2002). The cells expressed hepatocyte transcription factors HNF1α, HNF4α and GATA4, but not functional markers such as albumin. The cells differentiated into hepatocytes, expressing markers such as AFP, alcohol dehydrogenase and albumin, in aggregate culture and into biliary epithelial and oval-like cells, expressing markers such as gamma glutamyl transpeptidase (GGT), HNF6, CD117 (c-kit) and Thy1, on matrigel. However, the induction on matrigel was not specific as hepatocyte markers were also observed.

Suzuki *et al.* have isolated potential hepatic stem cells from developing mouse livers using specific markers (Suzuki et al., 2002; Suzuki et al., 2000a). These cells were clonally cultured *in vitro* and produced both hepatocytes and biliary epithelial cells, while also maintaining a population of stem cells. Furthermore, when injected into recipient animals, they formed functioning hepatocytes and bile duct structures. When transplanted to the pancreas or duodenal wall, the cells produced pancreatic ductal or acinar cells or intestinal epithelia.

Liver stem cells with the cell surface marker profile $CD49f^{+}CD29^{+}CD117^{neg}CD45^{neg}TER119^{neg}$ were isolated using flow cytometric cell sorting by Suzuki *et al.*. CD49f and CD29 are α6 and β1 integrin subunits, CD45 is a leukocyte common antigen and TER119 is exclusively expressed on immature erythroid cells. To enrich in progenitor cells even further, c-met (HGF-receptor) was used for a second round of flow cytometric cell sorting. C-met has an essential role in the development of the mouse liver. Six populations were created based on the level of expression of c-met and CD49f. The highest number of potential stem cells was found in the fraction $c\text{-}met^{+}CD49f^{+/low}$. These cells were cultured either on laminin or type IV collagen coated tissue culture plates. The culture media contained HGF and EGF and when cultured clonally, 50% of conditioned media from non-sorted foetal liver cells was also used. In culture, these cells differentiated to hepatocytes (expressing albumin) and biliary epithelial cells (expressing CK19) or expressed markers of both or neither. The cells that expressed neither gave rise to relatively large colonies (termed hepatic

colony-forming unit in culture, H-CFU-C, by the authors). Interestingly some of the cells started to express CD117, CD34 and Thy1, which are markers of oval cells in the adult liver. The authors suggest that the H-CFU-C cells differ from hepatoblasts in that they do not express lineage markers and are much rarer in the developing liver.

Minguet *et al.* derived a $CD117^{low}CD45^{neg}TER119^{neg}$ cell population from day 11 mouse embryo liver cells (Minguet et al., 2003). These cells expressed hepatospecific genes and proteins, were self-maintained *in vitro* by long-term proliferation and simultaneously differentiated into functional hepatocytes and bile duct cells. By flow cytometric analysis cells expressed low levels of c-met and either high or low CD49f expressing cells could be distinguished. Analysis of mRNA levels demonstrated the expression of albumin, AFP, TTR, HGF, OSM-receptor and c-met. The CD117 expression was rapidly downregulated after 24-48hrs in culture. HGF and OSM were used to differentiate the cells.

DLK (Delta-like or Pref-1) was identified as a potential marker of hepatoblasts by Tanimizu *et al.* by investigating mouse foetal liver (Tanimizu et al., 2003). DLK is a transmembrane protein containing epidermal growth factor-like repeat motifs. When cultured with HGF and EGF, the DLK^{+} cells expressed albumin, AFP and CK19. It was also shown that the cells differentiated into hepatocytes when transplanted into the mouse spleen.

In humans a potential liver progenitor population was isolated by Malhi *et al.* from foetal livers (Malhi et al., 2002). The progenitor liver epithelial cells expressed a combination of genes associated with hepatocytes, bile duct cells, oval cells and hepatoblasts, including AFP, GGT, CK8, CK19, CD34 and plasminogen activator inhibitor 1 (PAI-1). The cells were capable of differentiating into mature hepatocytes and of integrating into the liver parenchyma of SCID mice. Furthermore, the cells expressed telomerase. The cells could be grown on normal tissue culture plastic with no additional growth factors, lipids or extracellular matrix. These foetal cells expressed liver genes despite extensive culture, which is encouraging when compared to the rapid loss of function of primary adult hepatocytes in culture.

It has been shown that foetal mouse and rat hepatocytes can proliferate and mature to an adult phenotype when injected into adult mice or rats and that these cells proliferated more when normal hepatocyte replication was inhibited or when transplanted cells had a survival advantage (Cantz et al., 2003; Dabeva et al., 2000; Sandhu et al., 2001). However, proliferation was also seen in non-injured normal liver (Sandhu et al., 2001).

Nowak *et al.* injected $CD117^{+}CD34^{+}Lin^{neg}$ cells isolated from human foetal liver into a acute liver injury mouse model. The cells differentiated into hepatocytes and cholangiocytes. The cells expressed albumin, AAT, AFP, CK19, G6Pase, glycogen and GGT (Nowak et al., 2005).

Interestingly, foetal pancreatic cells have recently been shown to trans-differentiate into liver cells. This had been shown in a rat pancreatic cell line AR42J-B13, and was reported in organ cultures of pancreatic buds from mouse embryos when cultured with the glucocorticoid dexamethasone (Tosh et al., 2002a). This trans-differentiation is thought to be due to the close development of liver and pancreas during embryogenesis. The cells expressed albumin, transferrin, TTR (Tosh et al., 2002a) and glucokinase, P450s (CYP3A1 and CYP2B1/2) (Marek et al., 2003), testosterone/4-nitophenol uridine diphosphate glucuronosyltransferase, aryl sulfotransferase and enzymes involved in ammonium detoxification. These are enzymes that are strictly compartmentalised in the liver and this shows that both periportal and perivenous hepatocytes are induced (Tosh et al., 2002b). The cells were also transduced with GFP under the TTR or G6Pase promoter, and when the cells were cultured with dexamethasone, they started expressing GFP (Tosh et al., 2002b). The transcription factor 'CCAAT enhancer binding protein β' [C/EBPβ] was found to be responsible for the differentiation programme (Shen et al., 2000). Moreover, wild–type adult mouse pancreatic cells transplanted into FAH-/- recipients have also been able to rescue the mice and provide normal liver function (Wang et al., 2001).

1.6 Stem cells derived from umbilical cord blood and placenta

Umbilical cord blood (UCB) and placenta are other sources of stem cells. The isolation of these cells does not have ethical issues as it causes no harm to the newborn and the cells are readily available.

Danet *et al.* identified a rare human stem cell population from human cord blood (and bone marrow) expressing the receptor of the complement molecule C1q (C1qRp) (Danet et al., 2002). This $CD45^{+}CD38^{neg}CD34^{+/-}C1qRp^{+}$ cell population has haematopoietic (bone marrow repopulation) and hepatic potential (differentiated to mature hepatocytes in vivo).

Some UCB isolated stem cell populations ($CD34^{+}$ and $CD34^{neg}$) have been differentiated *in vitro*. Kakimuna *et al.* isolated hepatic progenitors and cultured them with HGF, FGF-1, FGF-2, LIF and SCF (Kakinuma et al., 2003). The cells started expressing albumin (50% of total cells) and had hepatic progenitor characteristics. When transplanted into murine models the cells differentiated into functional hepatocytes, although at low frequencies (0.1-1%). Haematopoietic and mesenchymal stem cells have also been isolated from UCB, and haematopoietic stem cells have been used successfully in haematopoietic reconstitution.

Nonome *et al.* cultured human UCB cells on collagen with HGF and FGF and showed liver specific gene expression after 7 days. Immunocytochemistry showed AFP, CK19 and albumin expression. *In vivo* studies in NOD-SCID mice showed human albumin, hepatocyte specific antigen positive cells in the Fas-mediated liver injury model. However, no difference between $CD34^{+}$ and $CD34^{neg}$ cells isolated from cord blood was seen (Nonome et al., 2005).

In vivo, human UCB stem cells have been found to differentiate into hepatocytes in a model of hepatic damage induced by allyl alcohol in NOD-SCID mice (Di Campli et al., 2004) and to generate CK18-negative hepatocyte-like cells in CCl_4 injured NOD-SCID mouse liver (Sharma et al., 2005). Cells in the latter study expressed human albumin and HepPar1 but mouse CK18 which, reflecting their co-culture with mouse cells, suggested the formation of chimeric hepatocytes *in vitro*.

Miki *et al.* have isolated stem cells from human amniotic epithelial cells isolated from term placenta following live birth (Miki et al., 2002). The amniotic tissue was found to stain for CK18, CK19, AAT, CD117 but not CD34, Thy1 or albumin. In culture these cells expressed CK18, CK19, Thy1, CD117, AAT and albumin. Flow cytometric analysis showed that the cells isolated from placenta expressed ES cell markers SSEA-

3, Tra-1-60 and Tra-1-81. 20% matrigel coated plates and dexamethasone and insulin-transferrin-selenium containing media and phenobarbital were found to be the best conditions to upregulate liver specific gene expression (Miki et al., 2003).

Another source of ethically acceptable liver stem cells is the adult human liver. Diseased explants are thought to be highly enriched in activated liver stem cells. Rather than only having the capacity to differentiate into functional liver cells (as during development), these cells are thought to be geared into the regeneration of liver tissue.

1.7 Adult liver stem cells – Oval cells

As discussed earlier, a relatively quiescent stem cell compartment is thought to be present in most adult tissues and organs (Blau et al., 2001). These cells are responsible for maintaining normal tissue homeostasis and repair. During injury, these cells are activated to counteract any damage to the tissue or organ.

Liver cells have a unique capability to respond to injury and have a massive self-renewal capacity. Some models suggest that rodents are able to recover even from a 90% liver resection. The extensive capability to recover from loss of liver tissue is based on hepatocyte activation. The normally quiescent hepatocytes exit the G_0 phase and enter DNA synthesis in order to proliferate and regenerate the missing tissue. In a rodent model of 2/3 partial hepatectomy (PH), proliferating cells are observed randomly across the liver and the liver is replaced within two weeks. Hepatocyte transplantation has also shown that the transplanted cells are capable of significant clonal expansion in the recipient liver (Alison et al., 2001; Kountouras et al., 2001; Alison et al., 1998b). Moreover, hepatocytes have also been able to function as a facultative stem cell in order to restore biliary epithelium during injury when biliary proliferation in compromised by biliary toxin methylene diamiline (Michalopoulos et al., 2005).

However, when the hepatocytes are unable to proliferate, for example in the presence of chemical carcinogens, the stem cell compartment is activated. When using the rodent model of PH in the presence of 2-acetylaminofluorene (2AAF) or carbon tetrachloride (CCl_4), it is seen that hepatocytes are slow to recover and another type of cell, termed oval cell, is activated and proliferates extensively. These oval cells are capable of

migrating into the liver parenchyma and differentiating into hepatocytes or biliary epithelial cells, i.e. they are bipotential (Alison et al., 2001; Kountouras et al., 2001; Alison et al., 1998b).

Several review papers discuss the activation and origin of oval cells in animal models and human disease (Alison et al., 1998b; Alison and Sarraf, 1998; Alison, 1998; Forbes et al., 2002; Oh et al., 2002; Sigal et al., 1992; Faris et al., 2001; Zhang et al., 2003b). This section will give an overview of the current knowledge of adult liver stem cells and oval cells.

1.7.1 What is the origin of oval cells?

There are several theories regarding the stem cell compartment in the liver. Liver stem cells are considered to be the progenitors of oval cells, but their origin is not known. It has been proposed that liver stem cells could arise from quiescent liver stem cells resident in the liver (notably comprising cells of the Canals of Hering (Theise et al., 1999)), or attracted from extrahepatic sources (e.g. bone marrow) during liver injury. Adult HSCs and mesenchymal stem cells have been known to differentiate into hepatocytes. However, it is uncertain if HSCs transdifferentiate into or fuse with resident hepatocytes. The HSCs as liver stem cells are discussed in more detail later in section 1.8.

1.7.2 What are oval cells?

Regardless of the origin of liver cell progenitors, oval cell activation is observed in severe liver disease when hepatocytes are unable to proliferate. When activated, they proliferate extensively to form ductules continuous with interlobular bile ducts which are termed 'reactive ductules' (Zhang et al., 2003b). The cells are bipotential, capable of differentiating into hepatocytes or biliary epithelial cells (Oh et al., 2002; Forbes et al., 2002; Zhang et al., 2003b). Oval cells are named after their morphology. The cells have oval nuclei and a high nucleus to cytoplasm ratio (Oh et al., 2002; Zhang et al., 2003b). What distinguishes oval cell activation from bile duct hyperplasias is that the cells are able to differentiate into mature hepatocytes (Alison et al., 1998b; Alison and Sarraf, 1998).

In humans the reactive ductules are surrounded by activated mesenchymal cells (myofibroblasts and Ito cells). These Ito cells acquire a myofibroblast-like phenotype expressing desmin and alpha-smooth muscle actin (α-SMA) during liver injury. The Ito cells provide the extracellular matrix (ECM) proteins during hepatic regeneration and also secrete proteinases. These functions may be vital for initiating oval cell proliferation and migration into the damaged parenchyma, even though oval cells also migrate along the space of Disse. The Ito cells may also insulate oval cells from the hepatocyte microenvironment during the migration to prevent early differentiation. (Alison et al., 1998b).

Even though it is recognised that oval cells are bipotential progenitor cells, it is uncertain if they can be regarded as stem cells or if they are partly differentiated or differentiated stem cell progeny. The 'stem cell degree' of many isolated or identified populations has not been concluded and the regeneration generated by stem cells is not fully understood. Furthermore, it is quite likely that different diseases will have different activation mechanisms and maybe even different compartments or cells are activated at different instances.

1.7.3 Where are oval cells located?

Oval cells are thought to be activated from the portal tract, canals of Hering or from blast-like cells next to bile ducts (Alison et al., 1998b; Alison and Sarraf, 1998; Alison, 1998; Zhang et al., 2003b). The canal of Hering represents the anatomic and physiological link between the intralobular canalicular system of the biliary tree. The canal of Hering continues into a channel lined by cholangiocytes, a ductule. The ductules link to the smallest interlobular bile ducts (Roskams et al., 2004).

In the small interlobular bile ducts of rats, 'blast-like' cells have been identified that are enveloped by ductular cells rather than in contact with the basal lamina or the lumen (Alison et al., 1998b). These cells are small with a dense heterochromatic nucleus and lack the expression of any differentiated markers. However, the smallest units of the biliary tree (canals of Hering, cholangioles, terminal ducts, ductules) proliferate extensively radiating from the portal tract and differentiate to hepatocytes (Alison et al., 1998b; Alison and Sarraf, 1998; Forbes et al., 2002). These cells share unique tight junctions both with hepatocytes and ductular cells (Faris et al., 2001). It is uncertain if

these cells which are scattered within the biliary tree are multipotent stem cells or lineage specific stem cells.

1.7.4 How are oval cells recognised?

Oval cells in humans are not completely characterised and are categorised by the expression of a combination of markers (Alison et al., 1998b; Alison and Sarraf, 1998). These markers are not oval cell specific, but some are highly expressed by oval cells. The markers include foetal liver, common stem cell and liver markers. Most of these markers have been identified by immunohistochemistry.

The original theory was that the oval cells are the progeny of hepatic adult stem cells, native to the liver. During development, a set of transitional cells are thought to remain in the adult liver as bipotential progenitors. When activated by liver injury to replace the damaged tissue, the cells express foetal liver markers, such as alpha-fetoprotein (AFP) normally expressed only in the developing liver.

Liver stem cells express markers associated with other stem cells. Markers associated with HSCs and expressed on liver stem cells include Thy1, Flt-3, CD34, CD117 (c-kit), its ligand stem cell factor (SCF) and functional stem cell markers such as Hoechst dye efflux.

Because of their location/origin oval cells also express biliary epithelial markers, for example GGT, CK7 and CK19. These cytokeratins are often expressed in combination of hepatocyte cytokeratins CK18 and CK8. CK14, a putative stem cell marker, has also been seen occasionally. Sometimes CK14 is expressed in combination with AFP (Alison et al., 1998b). OV-6, a rat oval cell marker, identifies a cytokeratin with epitopes shared on CK14 and CK19. In human liver, OV-6 is not oval cell specific but is found on cells in the ductal plate, oval cells, bile ducts, ductules in foetal tissue and oval cells found in focal nodular hyperplasia.

At the moment, there are no firm conclusions as to what markers should be used to look at human liver stem cells and hepatocyte progenitors.

1.7.5 Oval cells in animal models

Oval cells have been identified in rat models as long strings of ductular cells spreading outwards from the portal areas (Alison et al., 1997). These cells express a combination of CK8, CK19, vimentin and AFP (Alison et al., 1997) or OV-6, albumin, CK19 and CK7 (He et al., 2003) or Thy1, together with AFP, GGT, CK19, OV-6 and OC2 (oval cell antigen 2) (Petersen et al., 1998). Diethyldithiocarbamic acid (DDC) induced mouse model oval cells express the A6 marker, AFP and haematopoietic markers stem cell antigen-1 (Sca-1) and CD34 and CD45 (Petersen et al., 2003).

The cells then differentiate into hepatocyte-like cells lacking oval cell markers but expressing mature hepatocyte markers such as albumin, CK18, tyrosine aminotransferase, AAT and urea synthesis (Alison et al., 1997; He et al., 2003). Oval cell proliferation can be enhanced *in vivo* by HGF (Shiota et al., 2000) and by α-1-adrenoreceptor antagonists (in a choline-deficient diet model) (Oben et al., 2003). Pulse-chase experiments have demonstrated differentiation of oval cells into hepatocytes as well as biliary cells *in vivo* in a rat model (Evarts et al., 1996).

The dye-efflux phenotype of stem cells was utilised by Wulf *et al.* to isolate side population (SP) cells from mouse liver (Wulf et al., 2003). These mononuclear cells were small, blast-like cells with agranular cytoplasm. The majority were $CD45^+$ and distinct subpopulations of CD34, CD117, Sca-1 and Thy-1 positive cells were observed. In DDC-treated mice the cells contributed to liver regeneration (Wulf et al., 2003).

Small hepatocytes which are thought to be distinct from oval cells have also been reported in retrosine-induced hepatocellular injury models of rats (Gordon et al., 2000b; Gordon et al., 2000a; Gordon et al., 2002). These cells arose in any area of the hepatic lobule. The cells expressed OC.2 and OC.5 (oval cell/bile duct antigens) but not CD34 or Thy1. They expressed AFP and liver associated transcription factors, but not CYP450 (maybe therefore avoiding retrosine toxicity). The cells could not be maintained *in vitro*. The cells inserted into hepatic cords after transplanatation (Gordon et al., 2000b; Gordon et al., 2000a; Gordon et al., 2002).

Similar cells have been observed by Mitaka *et al.* (Mitaka et al., 1998). These small hepatocytes (1/3 – ½ size of a normal hepatocytes) were identified in culture and

thought to be originators of hepatocyte clones. The cells expressed albumin, transferrin CK8 and CK18 and after 10 days of culture they differentiated into mature hepatocytes (tryptophan 2,3 dioxygenase and connexion-32 expression) (Mitaka et al., 1998; Mizuguchi et al., 2001).

1.7.6 Oval cells in human disease

In human disease, oval cell activation has been investigated from patients with genetic hemochromatosis, alcoholic liver disease and chronic hepatitis C (Lowes et al., 1999) and in normal human livers and from patients with end-stage primary biliary cirrhosis and sclerosing cholangitis (Crosby et al., 1998). It was found that oval cell numbers were dependent on disease severity, rather than the type of disease (Lowes et al., 1999). Moreover, it was found that the more centrally located the progenitor cells were, the more severe the parenchymal inflammation was (Libbrecht et al., 2000).

The progenitor cells were identified by morphology and M2-PK (embryonic form of pyruvate kinase), π-GST (also stained hepatocytes) or CK19 (also stained biliary epithelium) expression (Lowes et al., 1999) or OV-6 expression (Crosby et al., 1998). Crosby *et al.* found that HEA125 and CK19 stained bile ducts and ductules in normal liver and proliferating ductular structures in diseased livers, whereas OV-6 did not label normal liver but stained many proliferating ductular and periductular cells and lobular hepatocytes. This suggests that OV-6 could be a human oval cell marker, even though the staining patterns of OV-6 in rats and humans differ and the specific antigen for human OV-6 is not yet characterised. Xiao *et al.* observed small oval cells with scanty cytoplasm, high nucleo-cytoplasmic ratio, expressing CK7 and albumin in proliferated bile ductules at margins of regenerating nodules and in fibrous septa in hepatitis B patients (Xiao et al., 2003).

De Vos *et al.* reported the presence of small cells with bile duct-type cytokeratin profiles in chronic ductular reaction in periportal area. These cells could be divivded into three subsets: one with oval cell characteristics, one with bile-duct differentiation features and one with hepatocllular differentiation characteristics (De Vos and Desmet, 1992).

1.7.7 Oval cells in massive hepatic necrosis

If disease severity correlates with liver progenitor cell numbers, as suggested, a liver with massive hepatic necrosis would be ideal starting material for liver stem cell isolation. It was found that parenchymal cells in human liver with massive hepatocyte necrosis contained cells co-expressing CK19 with HepPar1, CD117 and AFP (Theise et al., 1999). Furthermore, CD117 staining of CK19 positive cells was present in ductular reactions and in occasional bile ducts. Some CK19 positive cells showed co-staining with HepPar1 and AFP.

A small population of cells expressing CK14 together with CD117, flt3 and CK19, but lacking expression of albumin and CD34 has also been found (Seki et al., 2003). The cells intermingle with biliary ductule-like structures. The CK14 and CD117 expressing cells were only found in patients with acute type of fulminant hepatitis with multilobular necrosis and not in patients with sub-acute fulminant hepatitis.

$CD117^{+}CD133^{+}CD34^{neg}CD45^{neg}tryptase^{neg}$ cells, which were 7-11μm with scanty cytoplasm have been observed by Craig *et al.* in ductules and by occasional solitary lymphoid blast-like cells and they may represent human oval cells (Craig et al., 2004b; Craig et al., 2004a).

1.7.8 Stem cells from liver in culture

For a bio-artificial liver device, the crucial aim is to grow isolated stem cells successfully in culture. There is therefore a requirement of culture conditions to maintain stem cell proliferative potential and other conditions to promote hepatocyte differentiation.

The problem with these studies is that there is significant variation in the data presented so far. Differences in the source of cells, diseases or disease models in both animals and humans, isolation methods, markers and growth patterns, have all been used. The cells have been grown for varying amounts of time and the proof of the cells being stem cells is different. What follows is an account of the different studies published to date.

1.7.9 Culture of liver stem cells from animal models

In rat, liver progenitor cells have been isolated using morphology by identifying proliferating endodermal-like cells with polyhedral bright borders (Yin et al., 2002a), and in mouse by aggregation potential, where cells were in hypoxic 2-hr suspension culture with constant agitation. Hepatocytes formed aggregates and 5% of proliferating epithelial cells were left. These epithelial cells expressed AFP, E-cadherin and albumin but were CK19 negative (Azuma et al., 2003).

When cultured on gamma-irradiated STO-feeder cells the endodermal-like cells expressed HSC and early hepatic markers (Yin et al., 2002a). However, when cultured without feeders, the markers disappeared and were replaced by mature hepatocyte markers. If cultured on matrigel, duct like structures and biliary epithelial markers were observed.

Wang *et al.* used a perfusion and centrifugation technique to yield mouse progenitors which proliferated and matured into hepatocytes, expressing oval cell antigens peaking at 2 weeks of culture with progressive increase in albumin expression (Wang et al., 2003a).

Rat cells have also been isolated using surface markers, oval cell antigens 2 and 3 (OC2 and OC3) (Brill et al., 1993) and Thy1.1 (Petersen, 2001). OC2 and OC3 cells expressing albumin and AFP were cultured in co-cultures of embryonic liver-specific stroma, on type IV collagen mixed with laminin and media with complex lipids, low calcium and supplemented with insulin like growth factor II and Granulocyte-colony-stimulating factor (GCSF) (Brill et al., 1993). Thy1.1 positive cells expressed CD34, Flt3 and CD117 and were grown on fibronectin and laminin coated plates supplemented with IL-3, SCF and Flt3-ligand for over 8 months. The cells had an epithelial morphology (Petersen, 2001).

He *et al.* cultured OV-$6^{+}$$CK19^{+}$$CK7^{+}$ and $albumin^{+}$ cells from rats for three months and maintained the expression of the markers. The cells were cultured on feeder cells and supplemented with HGF, EGF, SCF, LIF and hepatic stimulator factor (He et al., 2004).

Side population (SP) cells (effluxing Hoechst dye) isolated from mouse liver by Wulf *et al.* were cultured in haematopoietic stem cell supporting methylcellulose media to form distinct colonies (Wulf et al., 2003). The non-SP cells did not form colonies whereas cultures supplemented with EGF, TGF-α and nicotamidine seemed to inhibit progenitor growth.

A particular strain of GFP-transgenic mice that express GFP in liver endodermal cells (GFP was under the cytomegalovirus enhancer-β-actin promoter and globulin-poly-A-tail (CAG) promoter) has been used to isolate and characterise these cells from adult liver (Fujikawa et al., 2003). Side scatter^{low} cells, corresponding to non-parenchymal cells, included two populations with different GFP-expression patterns; low and high. CD45^{neg}TER119^{neg}side-scatter^{low}GFP^{high} cells were sorted and characterised. The cells expressed AFP and integrins α6 (CD49f) and β1 (CD29), but were CD117 and Thy1.1 negative. In culture these cells could differentiate into hepatocytes and biliary epithelial cells.

Potential liver stem cells have been isolated from adult porcine liver (Kano et al., 2000). The cells expressed albumin, AFP, transferrin, CK18, CK7 and c-met but not AAT, OV-6, GGT, CK19 or CK14. The cells differentiated into hepatocyte-like and biliary epithelial like-cells, with no distinct differentiation signals.

TWEAK (TNF-like weak inducer of apoptosis) has been found to stimulate oval cell proliferation in mouse liver and *in vitro* through its receptor Fn14 (Jakubowski et al., 2005). TWEAK had no effect on mature hepatocytes. Increased Fn14 expression in chronic hepatitis C and other human diseases relative to normal liver was also shown. Transgenic mice overexpressing TWEAK in hepatocytes caused periportal oval cell hyperplasia (Jakubowski et al., 2005). However, it is still uncertain if TWEAK induces both oval cells and biliary epithelial cell proliferation, due to the similar markers found on these cells (Fausto, 2005). Because cytokines all work simultaneously *in vivo*, it is important to understand how they can be used *in vitro*. For example, *in vitro* TNF stimulates both hepatocytes and oval cells, but in combination with interferon-γ, TNF inhibits hepatocyte replication but induces oval cell replication (Fausto, 2005).

1.7.10 Culture of human liver stem cells

Fewer studies have been published on potential liver stem cells isolated from human liver. Crosby *et al.* (Crosby et al., 2001; Crosby et al., 2002) isolated putative liver stem cells by isolating CD117 and CD34 positive cells from livers with primary biliary cirrhosis (PBC) and alcohol liver disease (ALD). The cells expressing CD117 or CD34 were located in the portal tracts surrounding bile ducts and sometimes CD117 positive cells were found integrated into bile ducts. A small number of cells were found to co-express CK19. Cells expressing the same antigens were also observed in normal human liver, but these cells were not found to co-express CK19. The CD117 and CD34 cells were isolated and cultured in media supporting biliary epithelial cells for 7 days. In culture the cells produced two morphologies expressing CK19 and CD31 (endothelial cell marker). This suggested that the cells were capable of differentiating into biliary epithelial cells.

Selden *et al.* isolated a colony expressing hepatocyte, biliary epithelial and stem cell phenotypic markers from a sub-acute hepatic failure explant (Selden et al., 2003). These cells were cultured in hepatocyte supporting media for more than six months. The colony expressed albumin, AAT, GGT, biliary glycoprotein (BGP), CK7, CK18, CK19, c-met, TGF-β II and Oct-4.

1.7.11 Complex issues relevant to oval cell culture

The microenvironment is very important for liver stem cell maintenance, activation and differentiation. Coleman *et al.* transplanted oval cells subcutaneously, and the cells formed highly aggressive and poorly differentiated tumours. However, when the cells were transplanted into the liver, they lost the malignant phenotype or became at least more differentiated (Coleman et al., 1993). Cell-to-cell and cell-to-extracellular matrix connections are important for retaining cell polarity and tissue histogenesis, gene transcription and tissue architecture (Alison et al., 1998b; Alison and Sarraf, 1998).

When trying to grow and differentiate liver stem cells *in vitro*, it is important to mimic the natural regeneration process in the liver. Growth factors are important in signalling and stem cell culture media is often supplemented with them (Oh et al., 2002; Alison, 1998; Alison et al., 1998b; Alison et al., 1998a). It is thought that the factors involved in normal hepatic regeneration and stem cell mediated regeneration are the same. There

are at least three mitogens associated with regeneration, HGF, EGF and TGF-α. Oval cells have receptors for all these factors. These growth factors act as mitogens for primary hepatocytes in culture and HGF is a morphogen during normal liver development. HGF and SCF expression have been shown to increase in rodent models with liver injury, which then cause oval cell activation. HGF has been used to differentiate HSCs into liver cells. HGF also acts as a mitogen for liver stem cells derived from murine foetal tissue (Suzuki et al., 2003).

1.8 Other sources for adult liver cells

As already discussed, the origin of adult liver stem cells is uncertain. It has been suggested that liver stem cells are remnants of foetal cells, intrinsic liver stem cells or derived from circulating haematopoietic stem cells (HSCs). Moreover, mesenchymal stem cells isolated from human adult bone marrow have also been shown to be capable of differentiating into hepatocytes. This section introduces HSCs and mesenchymal stem cells as examples of adult stem cells, where important stem cell markers and culture requirements have been identified. Furthermore, their role in liver regeneration is discussed.

1.8.1 Haematopoietic stem cells

1.8.1.1 What are haematopoietic stem cells?

Szilvassy has written a very comprehensive review on HSCs (Szilvassy, 2003). HSCs maintain all lymphoid and myeloid cells that include the blood, bone marrow, spleen and thymus. HSCs are the most studied adult stem cell and are thought to have the potential to differentiate into cells of non-haematopoietic origin, e.g. into liver, pancreas, heart, brain, lung, skin gastrointestinal tract and kidney (Szilvassy, 2003; Herzog et al., 2003).

HSCs are not quiescent but have a very slow growth cycle. However, they have an extremely high proliferation potential and a single cell in a mouse can reconstitute and maintain the entire lymphohaematopoietic system of an irradiated or immunocompromised host. This capability is now considered the only valid test for true HSCs (Szilvassy, 2003; Domen and Weissman, 1999; Wognum et al., 2003).

HSCs reside in the adult bone marrow, although low numbers are also migrating in the circulation (Szilvassy, 2003). Less than 0.1% of the haematopoietic cells in the bone marrow are pluripotent stem cells capable of long term proliferation and self-renewal. HSCs have also been found in non-haematopoietic tissues, such as skeletal muscle, kidney, lung, liver, heart, brain and small intestine in the mouse. The cells residing in the bone marrow can be mobilised into the circulation with a wide range of cytokines (interleukin [IL] 1, 6, 7, 8, 11, 12, 17, granulocyte-colony stimulating factor [GCSF], granulocyte-macrophage colony-stimulating factor [GMCSF], SCF, Flt3/flk2 ligand), chemokines (macrophage inflammatory protein-1α [MIP-1α], stromal cell derived factor-1 [SDF-1]) and adhesion molecule agonists (VLA-4 antibody and AMD3100 [for CXCR4]) (Szilvassy, 2003).

GCSF mobilisation in humans is very varied and might be due to some unknown genetic difference (Szilvassy, 2003). GCSF is thought to act intrinsically on cells of the bone marrow microenvironment and on haematopoietic cells. Following GCSF treatment, granulocytes release proteolytic enzymes, such as neutrophil elastase and cathepsin, which cleave vascular cell adhesion molecule 1 (VCAM-1) and other adhesion molecules that normally keep clonogenic cells in the bone marrow. These cells are thus released into circulation (Szilvassy, 2003).

1.8.1.2 How are haematopoietic stem cells identified?

Much research has been undertaken towards identifying surface markers on HSCs. There is a large literature about different combinations of markers capable of distinguishing HSCs that have the capacity to repopulate immuno-deficient mice. Szilvassy (Szilvassy, 2003) lists all markers associated with haematopoietic stem cells and progenitor cells. The most relevant markers are discussed below.

CD34 is the marker most commonly used to isolate HSCs and is expressed on 1-4% of the nucleated cells in normal human bone marrow and <0.1% in normal human peripheral blood (Szilvassy, 2003). It is often used in conjunction after 'lineage depletion', i.e. Lin^{neg} selection. Lineage markers are a variety of surface markers associated with terminally differentiated blood cells: red blood cells, T-cells, B-cells, natural killer cells, monocytes and granulocytes (using antibodies against Gr-1, Mac-1,

B220, CD4, CD8, NK1.1, TER119). Lineage depletion gives 20-500-fold enrichement of HSCs (Wognum et al., 2003).

Another important marker is CD133 (AC133), which is a transmembrane glycoprotein of unknown function, expressed on the majority of $CD34^+$ cells (although populations which are $CD34^{neg}CD133^+$ have also been observed). CD117 (c-kit), the receptor for SCF, is expressed on 2/3 $CD34^+$ cells and thought to be an important marker of HSCs (Bunting and Hawley, 2003; Wognum et al., 2003). Upregulation of CD117 is seen in terminally differentiating erythroid cells, but the marker is absent in all circulating mature blood cells (Szilvassy, 2003). Ratajczak *et al.* investigated human haematopoietic CD34 and CD117 subsets ($CD34^{neg}CD117^{neg}$, $CD34^+CD117^{neg}$, $CD34^+CD117^+$) and their ability to engraft in SCID mice and found that only $CD34^+CD117^+$ cells were able to engraft (Ratajczak et al., 1999).

Different markers are often used together to try and identify a well-defined population of cells with stem/progenitor activity. $CD133^+CD7^{neg}CD34^{neg}Lin^{neg}$ cells, for example, are found to be highly enriched in progenitor activity and can form $CD34^+$ cells in culture (Szilvassy, 2003). Bhatia *et al.* isolated human $CD34^+CD38^{neg}Lin^{neg}$ cells from bone marrow and cord blood. These cells were capable of reconstituting the entire haematopoietic system after transplantation into immune-deficient mice (Bhatia et al., 1997).

Between 0.1-0.5% of $CD34^+$ cells also express VEGRF2 (vascular endothelial growth factor receptor 2/KDR and Flk1 in mice) (Szilvassy, 2003; Wognum et al., 2003).

The HSCs isolated from murine models have similar (but not the same) surface markers associated with them. In murine models, it has been found that HSCs isolated from adult bone marrow are $CD117^+Thy1.1^{low}Lin^{neg}Sca\text{-}1^+$ or $CD34^{low/neg}$ $CD117^+$ $Sca1^+$ and Lin^{neg}. The HSCs express CD34 depending on the developmental stage and cell cycle status (Bunting and Hawley, 2003; Domen and Weissman, 1999).

In addition to surface markers, the efflux of certain dyes has been used to identify HSCs. Hoechst 33342 and Rhodamine 123 efflux has been associated with more primitive stem cells (Bunting and Hawley, 2003). Goodell *et al.* found that murine bone

marrow cells capable of effluxing Hoechst 33342 were enriched at least 1000-fold for *in vivo* reconstitution capability (Goodell et al., 1996). These cells are termed the side population (SP). The expression of ABCG2 (ATP-binding cassette [ABC] subfamily G member 2) has been shown to be correlated with the capability of effluxing the dyes (Scharenberg et al., 2002; Zhou et al., 2001). ABC transporter superfamily proteins are transmembrane proteins involved in energy-dependent transport of substrates across membranes.

Moreover, aldehyde dehydrogenase (ALDH) activity has been used to isolate primitive human haematopoietic progenitors from cord blood (Storms et al., 1999). The enzyme is involved in oxidation of intracellular aldehydes and is thought to have an important role in oxidation of alcohol and vitamin A and in chemoresistance. Storms *et al.* developed a fluorescent substrate for ALDH (termed BAAA) and cells with low side scatter (SSC, cell granularity) and ALDH bright could be isolated in the presence of verapamil (multi-drug resistance inhibitor). 40-90% of the cells were found to be $CD34^{+}CD38^{low/-}$ and 50-100-fold enrichment in short and long-term culture assays (Storms et al., 1999).

1.8.1.3 Haematopoietic stem cells in culture

HSCs can be grown by two assays, clonogenic and expansion, choice of which is dependent on the experiments required of the cells. In the clonogenic assay the cells are grown in methylcellulose, which is a semi-solid media. This media allows single HSCs to form individual colonies. Cytokines and growth factors are also added (SCF, GM-CSF, IL-3, IL-6, GCSF, erythopoietin). For expansion, cells are cultured in a base liquid medium containing bovine serum albumin, human insulin, human transferrin, IL-3, IL-6, SCF and a reducing agent such as 2-mercaptoethanol. Some differentiation seems to occur and to address this Antonchuk *et al.* have transduced mouse bone marrow cells with transcription factor *Hoxb4* and were able to expand mouse HSCs 1000 fold (Antonchuk et al., 2002).

Ramsfjell *et al.* set up distinct growth requirements for optimal growth and expansion of human $CD34^{+}CD38^{neg}$ primitive bone marrow progenitor cells (Ramsfjell et al., 1999). These cells are normally quiescent but proliferated in response to early acting cytokines, SCF, flt3 ligand and megakaryocyte growth and development factor (MGDF). Omori *et*

al. showed that the flt3ligand/flt3 and SCF/CD117 signal transduction system can synergize and stimulate proliferation of HSCs (Omori et al., 1997).

Many feeder layers have been developed to support growth of haematopoietic stem cells, but Gupta *et al.* have shown that human haematopoietic stem cells can be maintained in stroma-free culture if supplemented with O-sulphated heparin sulphate glycosaminoglycans, IL-3 and either MIP-1α or platelet factor 4 (PF4) (Gupta et al., 2000).

1.8.1.4 HSCs can contribute to liver regeneration by differentiation or fusion

There are several important papers suggesting that haematopoietic stem cells contribute to the regeneration of liver cells after acute injury and also when no injury is present. This was firstly shown by bone marrow and liver transplant studies where bone marrow cells (which could be traced due to markers associated with the transplants) were found in the liver.

Petersen *at al.* was the first to suggest that bone marrow stem cells take part in the generation of hepatocytes, biliary epithelial cells or oval cell populations (Petersen et al., 1999). They used a rat model with partial hepatectomy or carbon tetrachloride and 2-acetylaminofluorene [2-AAF]. 2-AAF was used to inhibit hepatocyte proliferation. Bone marrow and whole liver transplantations were carried out in a cross-sex and cross-strain manner, so that the origin of the repopulating cells could be traced. In all of the rats, cells of bone marrow origin were found in the liver, suggesting that liver cells could be derived from the bone marrow.

Two groups have investigated the role of human HSCs in liver regeneration using transplant studies. Alison *et al.* looked at livers of female patients that had received a male bone marrow transplant and male patients who had received a female donor liver (Alison et al., 2000). Y-chromosome positive hepatocytes should indicate that an extrahepatic source, e.g. bone marrow, had contributed to liver regeneration. It was found that 0.5-2% of the hepatocytes of the diseased livers were Y-chromosome positive.

Another study by Theise *et al.* investigated human archival autopsy and biopsy samples from two female recipients of bone marrow transplants from male donors, and 4 male recipients of liver transplants from female donors (Theise et al., 2000b). Y-chromosome positive hepatocytes and cholangiocytes could be observed in all samples ranging from 4-43% and 4-38% respectively in the specimens. The highest numbers were from transplant recipients with recurrent hepatitis C. These results showed that large numbers of liver cells can be acquired from extrahepatic stem cells. The degree of contribution of HSCs into human liver is variable and is probably due to the severity of parenchymal damage (Theise et al., 2000b; Alison et al., 2001).

Another approach to the bone marrow and liver transplant studies has been direct transplantation of HSCs. Theise *et al.* showed that bone marrow cells contributed to the liver even without severe injury (Theise et al., 2000a). Lethally irradiated female mice were given $CD34^{+}Lin^{neg}$ cells from male donors. The results showed that up to 2.2% of the hepatocytes were Y-chromosome positive, indicating that the cells were bone marrow derived (also shown by bone marrow transplants). The irradiation of the animals before transplantation did not cause severe acute injury to the liver and therefore this study concluded that bone marrow cells were able to integrate into normal liver even without the regeneration of liver tissue.

The most interesting study used the FAH-/- mouse strain which is a model of tyrosinaemia. In this model the hepatocytes can be protected from toxic effects of endogenously generated succinylacetone by the administration of NTCB (2-(2-nitro-4-trifluoro-methyl-benzoyl)-1,3 cyclohexanedione), which blocks the metabolic pathway upstream of the enzyme defect and prevents the generation of the toxic metabolites. However, the removal of NTBC exposes the hepatocytes again to the toxin *in vivo*. This model was used to investigate hepatocyte transplantation, where transplanted wild-type hepatocytes with intact FAH activity had striking advantage over FAH-/- cells (Overturf et al., 1996). Lagasse *et al.* used purified HSCs ($Sca\text{-}1^{+}Thy1^{+}CD34^{+}lin^{neg}CD45^{+}$) from adult bone marrow and injected them intravenously into FAH-/- mice. The mice were rescued with restored biochemical function of the liver (Lagasse et al., 2000). Furthermore, engraftment has been shown even when no selection pressure was present (i.e. with NTBC) and that the cells proliferated (one cell to a nodule) when selection pressure was applied by the removal of NTBC (Wang et al., 2002).

HSCs from human cord blood and bone marrow ($CD34^+$ or $CD34^+CD38^{neg}CD7^{neg}$) have been transplanted into immunodeficient mice with liver damage (CCl_4) and one month later human specific CK19 and albumin (mRNA and protein) was observed in the mouse liver (Wang et al., 2003c).

How do stem cells arrive in the liver? To answer this question, Kollet *et al.* looked at the homing of HSCs into the liver of NOD-SCID mice. SDF-1, which attracts human and murine progenitors, was found to be expressed by liver bile duct epithelium (Kollet et al., 2003). If the SDF-1 receptor CXCR4 is neutralised, homing and engraftment was abolished. The injection of SDF-1, on the other hand, increased homing and engraftment. The engrafted human cells were localised in clusters surrounding the SDF-1-expressing epithelial cells and differentiated into albumin-producing cells. Irradiation and inflammation increased SDF-1 levels. Hepatic injury induced MMP-9 activity, causing increased CXCR4 expression and SDF-1 mediated recruitment of HSCs to the liver. HGF, which increased after liver injury, promoted CXCR4 upregulation and SDF-1 mediated migration of $CD34^+$ progenitors. HGF acted synergistically with SCF (Kollet et al., 2003).

CXCR4 expression has also been seen in oval cells and is thought to be important in their activation as well as the 'second wave' of recruitment of haematopoietic cells (Hatch et al., 2002).

SDF-1 is also up-regulated in biliary epithelial cells of human inflammatory liver disease and the plasma levels of SDF-1 have been found to be significantly higher in patients with liver disease than in normal controls. Most liver-infiltrating lymphocytes express CXCR4 (Terada et al., 2003).

These studies appeared to suggest that trans-differentiation of bone marrow derived cells occurred. However, more recently several studies pointing towards cell fusion have been published.

The studies involving the FAH-/- model were shown to involve fusion. Wang *et al.* showed the transplanted bone marrow cells from female donors that showed hepatocyte function in male recipients in the FAH-/- model demonstrated diploid to diploid fusion

(chromosome number 80, XXXY) and diploid and tetraploid fusion (chromosome number 120, XXXXYY) karyotypes suggestive of fusion between donor and host cells (Wang et al., 2003d). The southern blot results of the repopulating hepatocytes in the liver were incompatible with simple differentiation. Moreover, Vassilopoulos *et al.* reported that in the FAH-/- model, where the mice were given FAH+/+ positive bone marrow cells, the regenerating nodules contained more mutant than wild-type FAH alleles and that their hepatocytes expressed both donor and host genes (Vassilopoulos et al., 2003). These results would be consistent with polyploid genome formation by fusion of host and donor cells. They also showed that the haematopoietic donor genome adopted a more hepatocyte specific expression profile after cell fusion (Vassilopoulos et al., 2003).

Terada *et al.* demonstrated that mouse bone marrow cells can fuse with embryonic stem cells *in vitro* spontaneously in the presence of IL-3 (Terada et al., 2002). The fused bone marrow cells can adopt the phenotype of the recipient cells and could be interpreted as de- or trans-differentiation. Ying *et al.* showed in a similar study that when cells taken from mouse brain were cultured with ES cells, the brain cells appeared to be reprogrammed. However, these cells turned out to be fusion products between brain cells and ES cells (Ying et al., 2002).

On the other hand, some reports have shown that fusion does not always occur. Newsome *et al.* reported that human cord blood could differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion (Newsome et al., 2003). Wang *et al.* investigated a murine model (treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine) and observed that bone marrow cells did not repopulate the liver but the oval cells had originated in the liver itself (Wang et al., 2003b).

1.8.1.5 *In vitro* differentiation of HSCs into liver cells

A number of studies suggest that HSCs can also be induced to liver cells *in vitro*. These studies avoid the issue of cell fusion and provide information about growth factors and cytokines involved with differentiation.

Cells isolated from human bone marrow ($CD34^+CD45^+$) (Fiegel et al., 2003a) and peripheral blood monocytes ($CD14^+CD34^+CD45^+$) (Zhao et al., 2003) were found to

differentiate into liver cells in the presence of HGF. Fiegel *et al.* cultured the bone marrow derived cells on a collagen matrix with HGF and showed that the $CD34^{+}$ cells expressed CK19 and albumin after 28 days of culture, whereas the $CD34^{neg}$ fraction did not (Fiegel et al., 2003a). Zhao *et al.* showed differentiation of peripheral blood monocytes into macrophages, epithelial cells, endothelial cells, neuron cells and liver cells (Zhao et al., 2003).

Saji *et al.* used mouse bone marrow cells cultured in a collagen gel with or without growth factors (EGF, HB-EGF, HGF, OSM, BMP-4, bFGF and FGF4). With HGF, OSM, bFGF and FGF4 the cells expressed albumin, but bFGF was found to be most effective (Saji et al., 2004). CK19 was expressed by cells treated with HGF, HB-EGF, OSM, BMP-4, bFGF and FGF-4, again bFGF most effective. The cells differentiated with bFGF were further analysed and CK18, AAT were detected on day 6 while albumin and tyrosine aminotransferase (TAT) appeared on day 18. AFP was expressed in freshly isolated bone marrow cells by RT-PCR. By immunocytochemistry 5% of bone marrow cells cultured in bFGF for 6 days stained for both albumin and CK18. Albumin was also secreted into the culture media. The liver-enriched transcription factors C/EBPα and C/EBPβ were expressed by freshly isolated bone marrow and remained constant with bFGF. On the other hand, HNF1α, HNF3β, HNF4α, GATA4 and GATA6 were not expressed in freshly isolated bone marrow but appeared after 6 days of culture with bFGF. HNF3α appeared on day 12 with bFGF (Saji et al., 2004).

Mouse haematopoietic cells have been found to convert into liver cells ($CK18^{+}CD45^{neg}alb^{+}$) within days when co-cultured with injured liver (or liver supernatant). In these experiments the haematopoietic cells were separated from the liver cells or supernatant by a membrane to exclude the possibility of cell fusion (Jang et al., 2004). These HSCs were transplanted into liver-injured mice and found to convert to hepatocytes, with liver function restored after 2-7 days post transplantation. The rate of engraftment increased with more severe liver injury.

Okumoto *et al.* showed that both HGF and co-culture with hepatocytes can differentiate rat bone marrow ($lin^{neg}Sca\text{-}1^{+}$) cells (Okumoto et al., 2003). With hepatocytes, HNF1α and CK8 expression was seen on day 3, AFP and albumin on day 7 and with HGF culture HNF1α was observed on day 3 and CK8 day 7. Sera from liver failure patients

(with non-parenchymal cells) has also been used to stimulate the *in vitro* trans-differentiation of bone marrow derived cells (Yamazaki et al., 2003).

It has been suggested that tissue specific stem/progenitor cells could reside in the bone marrow. Kucia *et al.* proposed that bone marrow is a source of various stem-cell chemoattractants and survival factors and provides an environment that attracts tissue-specific circulating stem or progenitor cells (Kucia et al., 2004). mRNA of several early markers for muscle (Myf-5, Myo-D), neural (GFAP, nestin) and liver (CK19, alpha-fetoprotein) has been detected in circulating peripheral mononuclear cells (Ratajczak et al., 2004). Furthermore, GCSF increased the level of expression of these markers. It is therefore possible that isolated HSCs contain pre-existing tissue-specific stem or progenitor cells (Ratajczak et al., 2004; Kucia et al., 2004).

Avital *et al.* found that β2microglobulin^{neg}/Thy-1^{+} cells isolated from rat and human bone marrow stained positive for liver markers albumin, AFP, CK8, CK18 and CK19 and transcription factors C/EBPα, CYP3A2 and HNF4. (Avital et al., 2001). β2microglobulin is expressed broadly on almost all nucleated cells in mammals, but some immortal cancer cell lines and the inner cell mass of blastula does not express it. Negative cells may thus represent progenitors (Wang et al., 2005). The cells lacked haematopoietic markers CD34, CD38 and CD117. These cells were also found to be increased in rat bone marrow with cholestasis. After co-culture with hepatocytes, these cells could synthesise urea and after transplantation into immunosuppressed rats they integrated into hepatic cords.

Miyazaki *et al.* observed that c-met, AFP, CD34, Thy1 and CD117 expressing cells isolated from adult rat bone marrow expressed albumin if cultured in the presence of HGF and EGF (Miyazaki et al., 2002).

1.8.1.6 Adult stem cell plasiticity confusion

Adult stem cell plasticity remains a controversial area. There are a large number of recent reviews and the arguments presented in these reviews are discussed below (Preston et al., 2003; Fang et al., 2004; Tosh and Strain, 2005; Theise, 2004; Dahlke et al., 2004; Alison et al., 2004a; Blau et al., 2001; Alison et al., 2004b; Forbes et al., 2002; Theise, 2004; Mezey, 2004).

It is now widely accepted that probably both HSC trans-differentiation (or trans-determination) and fusion occurs and that myelomonocytic cells (granulocytes) are the major source of hepatocyte fusion partners (Camargo et al., 2004). The amount of contribution and the relevance of such relatively small number of cells engrafting remains an issue.

Theise *et al.* showed 4-43% hepatocytes were of donor origin after bone marrow transplantation (Theise et al., 2000b), but other studies have shown that this number is often much lower and that other cells of donor origin are also present. Ng *et al.* investigating post-transplantation liver biopsy specimens found most cells to have macrophage/kupffer cell differentiation (64-75%) and only around 1.6% cells showed hepatocytic differentiation. These hepatocytes made up only 0.62% of all hepatocytes (Ng et al., 2003). Korbling *et al.* looked at histological sections of the biopsy specimens and found 0-7% of the cells of donor origin in patient who had received haematopoietic cell transplantation from peripheral blood (Korbling et al., 2002). Hove *et al.* looked at cells of donor origin in recipient livers and found endothelial cells in 4/5 of the livers, bile duct epithelial cells in 3/5 and hepatocytes in only hepatocytes 1/5 of the livers (Hove et al., 2003). Moreover, female livers containing male cells have also been observed in patients who have not had bone marrow (or other) transplants. These cells are thought to be derived from foetal cells from pregnancy (Stevens et al., 2004). However, as male patients were included in the studies discussed earlier (and re-examination of the female patient pregnancy records were performed (Alison et al., 2000)), the data cannot be refuted, but this is something that should be taken into account.

Regarding HSC trans-differentiation/trans-determinantion, the evidence of their functionality as progenitors (clonal expansion of the engrafted cells) is lacking. However, this would be difficult to test as a marker of cell origin would be needed in order to show that the new lineage is derived from the old. Nevertheless, it is important to distinguish between the two scenarios where of (1) the cells engrafted adopt phenotypic traits of that organ (trans-differentiation) and (2) the engrafted cells become local stem cells able of clonal expansion (trans-determination) (Preston et al., 2003).

The remaining questions involve the physiological and therapeutic relevance of the HSC contribution. It is possible that a strong selection pressure for the use of HSCs is needed in the form (1) very severe liver injury and (2) an advantage of HSCs compared to any internal repair mechanism before this mechanism is activated. The HSCs might therefore be working as a back-up system when local stem cells are failing. Cells thought to be derived from fusion could rescue FAH-/- mice from death, so fusion events may also be therapeutically important.

Interestingly, another case of adult stem cell trans-differentiation has been observed by hepatic oval cells adopting morphology and antigenic phenotype of macroglial and microglial cells when transplanted to the neonatal mouse brain. The cells were functional with active phagocytosis (Deng et al., 2003).

1.8.2 Mesenchymal stem cells

1.8.2.1 What are mesenchymal stem cells, mesenchymal progenitor cells and multipotent adult progenitor cells?

Mesenchymal-derived adult stem/progenitor cells are introduced in this section as another source of useful information on surface markers and culture requirements of stem cells. These cells have also been shown to differentiate into liver cells *in vitro*.

Mesenchymal stem cells (MSCs), multipotent adult progenitor cells (MAPCs) and mesenchymal progenitor cells (MPCs) can be isolated from adult bone marrow and are distinct from haematopoietic stem cells. They support the growth of haematopoietic stem cells by providing bone marrow stroma (Roufosse et al., 2004). MSCs are also capable of differentiating into bone, cartilage, tendon, fat and skeletal muscle in rodents, (Bunting and Hawley, 2003; Pittenger et al., 1999). MAPCs can differentiate into all MSC-derived cell types and also have the capability to contribute to progeny of all three embryonic germ layers. *In vitro*, these cells have been seen to differentiate into hepatocyte-like cells and cells having characteristics of midbrain neurones (Bunting and Hawley, 2003; Schwartz et al., 2002). The MAPC have also been identified in other tissues, which suggests that the cells either migrate to other tissues, or that these cells can actually be isolated from different organs (Bunting and Hawley, 2003; Roufosse et al., 2004).

The mesenchymal stem/progenitor cells isolated from bone marrow are found to be heterogeneous in morphology and immunophenotype. The lack of specific antigenic markers has made it difficult to achieve robust methods of isolation and characterisations of these progenitors (Roufosse et al., 2004). The markers expressed and not expressed by the different cell subsets isolated from human bone marrow in the different studies are listed below.

The MSC cells isolated by Pittenger *et al.* (Pittenger et al., 1999) were SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a and CD124 positive and negative for CD14, CD34 and CD45. These cells could be induced to differentiate into adipocytic, chondrocytic or osteocytic lineages, and individual cell derived colonies remained multipotent.

Reyes *et al.* isolated human MPCs which had the phenotype $CD34^{neg}CD44^{low}CD45^{neg}CD117^{neg}$Class-I-$HLA^{neg}HLA\text{-}DR^{neg}$ (Reyes et al., 2001). The cells also expressed high levels of CD13 and CD49b. These cells were cultured for over 60 doublings on fibronectin in culture media supplemented with EGF and PDGF-BB. They could be cultured in serum free media if supplemented with Insulin-like growth factor-1 (IGF-1), although the growth rate was slowed. Differentiation was induced by the removal of these growth factors and the supplementation of others to produce osteoblasts, chondrocytes, adipocytes, stromal cells, skeletal myoblasts (limb-bud mesoderm), as well as endothelial cells (visceral mesoderm). When MPCs were plated on laminin or type IV collagen, cells expressed CD44 and class-I-HLA, a similar phenotype to some reports of MSCs, and proliferated poorly after 30 doublings. This suggests that the signalling via the $\alpha4\beta1$ or $\alpha5\beta1$ integrins is required to maintain the more primitive MPC phenotype.

MAPC isolated by Jiang *et al.* from murine bone marrow had the potential to differentiate into osteocytes, chondrocytes and adipocytes. The cells expressed Str1, CD13, CD49α and β, CD29, CD44, CD71, CD90, CD106 and CD124 (Jiang et al., 2002). When injected into early blastocysts they were able to contribute to most somatic cell types and in non-irradiated hosts they produced cells of the haematopoietic lineage as well as epithelium of liver, lung and gut.

Schwartz *et al.* have isolated human MAPCs from adult human bone marrow and differentiated these cells to functional hepatocytes *in vitro* (Schwartz et al., 2002). The cells were cultured on matrigel with FGF-4 and HGF. After seven days of culture the cells expressed hepatocyte nuclear factor 3β (HNF3β), GATA4, CK19, TTR and AFP. On days 14-28 the cells expressed CK18, HNF4 and HNF1α. The functional characteristics included urea and albumin secretion, uptake of LDL, glycogen storage and Phenobarbital-induced cytochrome p450 activity.

It has also been suggested that mesenchymal stem/progenitor cells can be found to circulate in peripheral blood, but there is no evidence to demonstrate that these cells will have a role in tissue repair (Roufosse et al., 2004). The cells have, however, been found in a wide range of tissues when injected into primates (Devine et al., 2003). In one study, MSCs were transduced with GFP and infused into three adult baboons. The tissues were analysed for transgene expression and MSCs were found in gastrointestinal tissues, kidney, lung, liver, thymus and skin with an estimated level of engraftment between 0.1% to 2.7% (Devine et al., 2003).

1.9 Conclusion

This introduction lays out the complexity of stem cell and liver cell biology. Although a large number of markers have been associated with liver stem cells, they are not consistent throughout the different studies. Table 1-2 shows a summary of the different markers expressed by the cells discussed in the introduction. In a similar manner, growth conditions and differentiation studies vary. Furthermore, a large amount of the work on liver progenitors has been performed in animal models, which is often difficult to relate back to human cells. This thesis aims to investigate different characteristics and markers that could be used to identify human liver stem/progenitor cells and their potential to be used in a bio-artificial liver device. The cells are isolated from diseased adult human liver, thought to be enriched in progenitors.

Table 1-2 A summary of markers expressed by cells observed in or derived from various sources which have the potential to develop into hepatocytes. Rodent studies are shown in italic and human studies are in normal text.

From embryonic stem cells:

- $SSEA1^{neg}SSEA3^{+}SSEA4^{+}$Tra-1-$60^{+}$Tra-1-$81^{+}Oct4^{+}hTERT^{+}CD133^{+}$ (Carpenter)
- *$SSEA1^{+}SSEA3^{neg}SSEA4^{neg}$ (Henderson)*
- Oct-4^{+} (Reubinoff) *(Nichols)*
- $Nanog^{+}$ (Mitsui and Chambers)

From foetal liver:

- β1 and α1,5,6,9 $integrin^{+}$ (Couvelvard)
- *$E\text{-}cadherin^{+}AFP^{+}alb^{+}$ (Nitou)*
- *$OX43^{neg}OX44^{neg}Thy1^{+}$ (Fiegel)*
- *$HNF1\alpha^{+}HNF4\alpha^{+}GATA4^{+}alb^{neg}$ (Strick-Marchand)*
- *$CD49f^{+/low}CD29^{+}CD117^{neg}$ $c\text{-}met^{+}CD45^{neg}TER119^{neg}$ (Suzuki)*
- *$CD117^{low}CD45^{neg}TER119^{neg}CD49f^{+/neg}alb^{+}AFP^{+}TTR^{+}HGF^{+}OSMR^{+}c\text{-}met^{low}$ (Minguet)*
- *DLK^{+} (Tanimizu)*
- $AFP^{+}GGT^{+}CK8^{+}CK19^{+}CD34^{+}PAI\text{-}1^{+}$(Malhi)

From cord blood and placenta:

- $C1rRp^{+}CD45^{+}CD38^{neg}CD34^{+/neg}lin^{neg}$ (Danet)
- $CD34^{+/neg}$ (Kakinuma and Nonome)
- $CK18^{+}CK19^{+}AAT^{+}CD117^{+}CD34^{neg}Thy1^{neg}alb^{neg}SSEA3^{+}$Tra-1-$60^{+}$Tra-1-$81^{+}$ (Miki)

From adult liver (Oval cells):

- *$CK8^{+}CK19^{+}CK14^{+}AFP^{+}vimentin^{+}$ (Alison)*
- *$OV\text{-}6^{+}alb^{+}CK19^{+}CK7^{+}$ (He)*
- *$Thy1^{+}AFP^{+}GGT^{+}CK19^{+}OV\text{-}6^{+}OC2^{+}$ (Petersen)*
- *$A6^{+}AFP^{+}Sca\text{-}1^{+}CD34^{+}CD45^{+}$ (Petersen)*
- *$SP^{+}CD45^{+/neg}$ and sub populations of $CD34^{+}CD117^{+}Sca\text{-}1^{+}Thy\text{-}1^{+}$ (Wulf)*
- M2-PK^{+}π-$GST^{+}CK19^{+}$ (Lowes)
- $OV\text{-}6^{+}$ (Crosby)
- $CK7^{+}alb^{+}$(Xiao)
- $CK19^{+}CD117^{+}$ and $CK19^{+}HepPar1^{+}AFP^{+}$ (Theise)

- $CK14^{+}CD117^{+}flt3^{+}CK19^{+}CD34^{neg}Alb^{neg}$ (Seki)
- $CD117^{+}CD133^{+}CD34^{neg}CD45^{neg}tryptase^{neg}$ (Craig)
- *$AFP^{+}E\text{-}cadherin^{+}alb^{+}CK19^{neg}$ (Azuma)*
- *$OC2^{+}OC3^{+}alb^{+}AFP^{+}$ (Brill)*
- *$Thy1^{+}CD34^{+}Flt3^{+}CD117^{+}$ (Petersen)*
- *$GFP^{+}CD45^{neg}$side scatter$^{low}TER119^{neg}AFP^{+}CD49f^{+}CD29^{+}CD117^{ne}gThy1^{neg}$ (Fujikawa)*
- *$Alb^{+}AFP^{+}transferrin^{+}CK18^{+}CK7^{+}c\text{-}met^{+}AAT^{neg}OV6^{neg}GGT^{neg}CK19^{neg}CK14^{neg}$ (Kano – isolated from porcine liver)*
- $CD117^{+}$ and/or $CD34^{+}$ (Crosby)
- $Alb^{+}AAT^{+}GGT^{+}BGP^{+}CK7^{+}CK18^{+}CK19^{+}c\text{-}met^{+}TGF\beta\text{-}II^{+}Oct\text{-}4^{+}$ (Selden)

From adult liver (Small hepatocytes):

- *$OC.2^{+}OC.5^{+}CD34^{neg}Thy1^{neg}AFP^{+}CYP450^{neg}$ (Gordon)*
- *Alb^{+} $transferrin^{+}CK8^{+}CK18^{+}$ (Mitaka)*

From bone marrow (hematopoietic stem cells):

- $C1rRp^{+}CD45^{+}CD38^{neg}CD34^{+/neg}lin^{neg}$ (Danet)
- *$CD34^{+}lin^{neg}$ (Theise)*
- *$Sca\text{-}1^{+}Thy1^{+}CD34^{+}lin^{neg}CD45^{+}$ (Lagasse)*
- $CD34^{+}CD38^{neg}CD7^{neg}$ and $CD34^{+}$(Wang)
- $CD34^{+}CD45^{+}$ (Fiegel)
- *$CK18^{+}CD45^{neg}alb^{+}$ (Jang)*
- *$Sca\text{-}1^{+}Lin^{neg}$ (Okomoto)*
- $\beta 2micro^{neg}Thy1^{+}Alb^{+}AFP^{+}CK8^{+}CK18^{+}CK19^{+}C/EBPa^{+}CYP3A2^{+}HNF4^{+}$ $CD34^{neg}CD38^{neg}CD117^{neg}$ *(Avital)* (Avital)
- *$c\text{-}met^{+}$ $AFP^{+}CD34^{+}Thy1^{+}CD117^{+}$ (Miyazaki)*

From bone marrow (mesenchymal stem cells):

- $SH2^{+}SH3^{+}CD29^{+}CD44^{+}CD71^{+}Thy1^{+}CD106^{+}CD120a^{+}CD124^{+}CD14^{neg}CD34^{neg}$ $CD45^{neg}$ (Pittenger)
- $CD34^{neg}CD44^{low}CD45^{neg}CD117^{neg}$Class-I-$HLA^{neg}$HLA-$DR^{neg}CD13^{+}CD49b^{+}$ (Reyes)
- $Str1^{+}CD13^{+}CD49\alpha^{+}CD49\beta^{+}CD29^{+}CD44^{+}CD71^{+}Thy1^{+}CD106^{+}CD124^{+}$ (Jiang)

From peripheral blood:

- $CD14^{+}CD34^{+}CD45^{+}$ (Zhao)
- $AFP^{+}CK19^{+}$ *(Ratajczak)* (Ratajczak) (Kucia)

1.10 Aims

Against the background presented in this chapter, the aims of the thesis are to:

1) Isolate proliferating populations of putative liver stem cells from human explant livers.
2) Characterise the isolated liver stem cell populations.
3) Culture stem cell populations long term.
4) Differentiate liver stem cells into functional liver cells.
5) Identify liver stem cell markers that can be used to select potential stem/progenitor cell populations.

1.11 Hypotheses

The specific hypotheses that will be addressed are:

1) Human explant livers are enriched in liver stem cells/hepatocyte progenitors which can be isolated from the non-parenchymal cell fraction by liver perfusion.
2) Liver stem cells express surface markers or combinations of surface markers that can be used to isolate specific populations of cells with stem cell potential.
3) The isolated progenitor cell populations can be grown long-term in culture because of the nature of stem cells.
4) Liver stem cells can be differentiated towards mature hepatocyte and/or biliary epithelial cells *in vitro*.

Chapter 2 - General Methods

2.1 Explant and normal liver samples

Human explant liver samples were acquired with patient consent (Ethics ID38-2000 Culture of human liver cells obtained at surgery) from livers removed during orthotopic liver transplants performed at the Royal Free Hospital. Only livers which were hepatitis A, B and C negative were used. A resection of the left lobe, weight ranging from 38 to 263g, was removed by a histopathologist. Samples were provided by Professor B. Davidson and co-workers, Department of Surgery, Royal Free Hospital. The different disease aetiologies included were alcoholic liver disease (ALD), primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), cryptogenic (cry), sub-fulminant (sub-ful) and fulminant (ful) liver failure, re-transplant and secondary biliary cirrhosis.

Normal human liver samples were acquired with patient consent (Ethics ID38-2000 Culture of human liver cells obtained at surgery) from specimens taken during liver resection for colorectal cancer performed at the North Hampshire Hospital. Only tissue considered 'normal' away from tumour margins were used. The weight of the tissue ranged from 29g to 69g. Samples were provided by Mr M. Rees, Department of Surgery, North Hampshire Hospital. These tissue samples have been designated the label BS.

2.2 Non-parenchymal cell isolation

2.2.1 Collagenase liver perfusion

The resected tissue was flushed with HBSS (Hanks' Balanced Salt Solution, Sigma cat noH9394) supplemented with 10mM HEPES (Gibco cat no15630-056) to remove blood and three veins in the tissue were identified. Cannulae tubing (size ranges: internal diameter from 0.5mm to 1mm and tube wall thickness from 0.5mm to 1mm) was glued into place with surgical tissue adhesive (Indermil, Tyco Healthcare cat no028830). The three veins were briefly flushed with HBSS/HEPES with 0.5mM EGTA to remove any calcium. Calcium removal decreases cell-to-cell contact prior to collagenase digestion.

The liver lobe was transferred into a plastic perfusion bag and placed in a 38°C waterbath. The cannulae were connected to a perfusion pump (Gilson Minipuls 3) and a

pressure tube to monitor the pressure during the recirculating perfusion. To bring the tissue to 37°C (the temperature required for optimal collagenase activity) and to remove chelating agent the liver was perfused with warm HBSS/HEPES for 10-15min depending on the size of the resection. The pressure was kept constant between 35 and 45cm H_2O. The HBSS/HEPES was siphoned out and replaced with 500ml DMEM media (GibcoBRL cat no32430–027 containing 264mg/l calcium chloride) with 250mg filter sterilised collagenase P (Roche cat no1213873). The lobe was then perfused for 15-35min, keeping the pressure constant between 35 and 45cm H_2O. The time of the perfusion was dependent on the state of cirrhosis of the tissue, i.e. the more cirrhotic the tissue, the longer the collagenase perfusion. The DMEM/collagenase solution was siphoned out and replaced with HBSS/HEPES for a further 2min flush.

Once the tissue was brought back to a Class II cabinet it was minced with scalpels. The chopped up tissue was then put through a metal strainer to remove clumps, and the sequentially through a 100μm and a 50μm mesh.

2.2.2 Separating parenchymal and non-parenchymal cells

The cell suspension was centrifuged for 2min at 50g at room temperature. The pellet contained the parenchymal cells and the supernatant the non-parenchymal cells. The non-parenchymal cells were put through a 30μm mesh to remove most of the remaining hepatocytes. The cells were centrifuged for 10min at 200g.

2.2.3 Freezing and thawing non-parenchymal cells

The non-parenchymal cell pellet was resuspended in 20ml proliferating media (PM, see 2.3.1) and placed on ice at a 45° angle. 20ml cold freezing mix (20% DMSO [Dimethyl sulfoxide, Sigma cat noD-5879] in 80% FBS [foetal bovine serum, HyClone cat no30160.03]) was then added dropwise while rotating the cell suspension slowly. The mixture was then aliquoted into 1.8ml cryogenic vials (Corning cat no2027), wrapped in cotton wool in a polystyrene box and left at -80°C overnight. The next day the cells were transferred into liquid nitrogen.

To recover the cells, the vials were quickly thawed in a 37°C waterbath. Warm complete DMEM (+ 10% FCS) was added into the vials, thereafter the cell suspension

was transferred into a 50ml test-tube and diluted up to 40ml with complete DMEM. The non-parenchymal cells were recovered by centrifugation for 10min at 200g.

2.2.4 Lymphoprep gradient

If cells were to be used fresh, a density gradient separation procedure (hereafter referred to as lymphoprep) was used to separate non-parenchymal cells from red blood cells, dead cells and platelets. The non-parenchymal cells were resuspended in 20ml of media and slowly layered on top of 20ml lymphoprep (Axis-Shield cat no1001967) at room temperature to achieve two distinct layers. The preparation was then centrifuged for 25min at 400g at room temperature. After centrifugation a band of non-parenchymal cells could be seen between the two supernatants [Fig. 2-1]. The red cells and dead cells pellet at the bottom of the tube. The non-parenchymal cells were collected and washed twice in DMEM media (400g and 200g for 10min respectively).

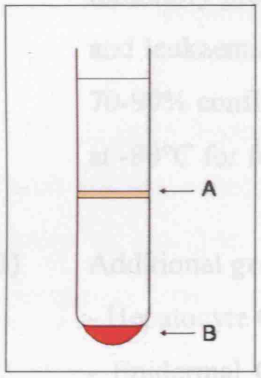


Fig. 2-1 A lymphoprep gradient was used to separate non-parenchymal cells from red blood cells and dead cells. The non-parenchymal cells are found as a layer in (A) and the red blood cells and dead cells pellet down to (B).

2.3 Non-parenchymal cell culture

The specific growth requirements of liver stem cells and progenitors are unclear so a combination of different media, growth factors and coatings were used. The freshly isolated cells were cultured in a humidified incubator at 37°C, 5%CO_2 with weekly 50% or 100% media changes. Between 50 and 200 wells were cultured from each liver sample. Cells were cultured at $1.5x10^5$ cells per well in 350µl medium in 48-well plates.

2.3.1 Culture media

1) **Proliferating media** (PM) based on Block *et al.* hepatocyte growth media (Block et al., 1996). Alpha-MEM (Gibco cat no22571-020) was supplemented with penicillin/streptomycin (50U/ml), fungizone (1.25μg/ml), 10% foetal bovine serum, D-glucose (25mM), glutamine (0.292g/L), thyrotropin-releasing hormone (0.04μg/ml), hydrocortisone (0.04μg/ml), insulin (1μg/ml), linoleic acid albumin (0.05mg/ml), sodium selenite (0.002μg/ml), ferrous sulphate (0.5mg/L), zinc sulphate (0.75mg/L) and nicotinamide (10mM).

2) **HS-5 conditioned media** used 1:1 with PM. HS-5 is a human bone marrow stromal cell line that supports haematopoietic stem cell growth (Roecklein and Torok-Storb, 1995). Growth factors secreted by HS-5 include Granulocyte colony-stimulating factor (GCSF), granulocyte-macrophage-CSF (GM-CSF), macrophage-CSF (MCSF), Kit-ligand (stem cell factor [SCF]), macrophage-inhibitory protein-1 alpha, IL-1alpha, IL-1beta, IL-1RA, IL-3, IL-6, IL-8, IL-11 and leukaemia inhitory factor (LIF). The conditioned media was collected from 70-90% confluent flasks and filter sterilised. The media was used fresh or stored at -80°C for future use.

3) Additional **growth factors**:
   - Hepatocyte Growth Factor (HGF) at 20ng/ml (PeproTech cat no100-39)
   - Epidermal Growth Factor (EGF) at 10ng/ml (R&D Systems cat no236-EG-200)
   - Fibroblast Growth Factor 4 (FGF-4) 5ng/ml (R&D systems cat no235-F4)
   - Platelet Derived Growth Factor (PDGF-BB) 5ng/ml (R&D systems cat no220-BB)
   - Granulocyte Colony Stimulating Factor (GCSF) at 1mu/ml (Neupogen, 30MU Filgrastim, Angen Europe BV cat no7028800)

   Concentrations used were according to manufacturer's suggestions.

2.3.2 Culture plates and coatings, feeder layers and conditioned media

Normal tissue culture plastic (Nunc cat no150628, 143982, 150687, 167008) was used for culture and also for growing feeder layers and fibronectin coating.

2.3.2.1 Fibronectin coating

50ug/ml fibronectin (Roche cat no 1080 938) was plated at 100μl/cm^2 onto tissue culture plastic and incubated at room temperature for 45min. The liquid was carefully removed after incubation and the plates were used for culture with normal culture media PM.

2.3.2.2 HS-5 feeder cells

HS-5 (ATCC cat no CRL-11882) human bone marrow stromal cells were used as a feeder layer and to collect conditioned media.

2.3.2.2.1 HS-5 cell culture

HS-5 cells were cultured in DMEM media (Gibco cat no31966-021 supplemented with 10% FBS, penicillin/streptomycin and fungizone) in a humidified incubator at 37°C, 5% CO_2. The media was changed every 3-4 days. For subculture the cells were trypsinised at 70-80% confluency. Centrifugation was carried out at 125g for 4min.

2.3.2.2.2 HS-5 feeder layers

Irradiated feeder cells were gamma-irradiated at 5Gy/min in suspension for 30s-4min. Native or irradiated HS-5 cells were plated into 48-well tissue culture wells at the appropriate density to achieve a 50% confluent well.

2.3.2.2.3 HS-5 conditioned media

Conditioned media was collected from 70-80% confluent monolayers. The conditioned media was filtered through a 0.2μm filter and frozen at -80°C.

2.3.3 Sub-culture - Trypsinisation

Non-parenchymal cells and all adherent cell lines were trypsinised using the same method. The culture media was removed and the cell monolayer was washed 2-3 times with HBSS (Mg^{2+} and Ca^{2+} free) to remove any remaining FBS. The cells were then incubated with 0.25% trypsin-EDTA (Life Tecnologies, cat no35400027) for 1-2min or until the cells detached. Fresh media (containing FBS) was added to the cell suspension to inactivate the trypsin. The cells were then centrifuged, resuspended in fresh media, counted [2.3.3.1] and reseeded at an appropriate density. If very small numbers of cells

were trypsinised, they were not centrifuged, but reseeded directly after the inactivation of the trypsin.

2.3.3.1 Viable cell count by trypan blue exclusion

The viable cell count was performed by trypan blue exclusion. An aliquot of the cells was diluted (1:10) into HBSS (8:10) with trypan blue (1:10) (0.2% Trypan blue in PBS) and kept at room temperature for 2min. The cells were then counted with a haemocytometer. The live cells will exclude the dye, whereas the dead cells have nuclei stained blue.

2.4 Positive and negative controls

2.4.1 Cell lines

A number of cell lines and human peripheral blood samples were used as positive and negative controls for different methods, i.e. immunocytochemistry, RT-PCR, flow cytometry.

2.4.1.1 MO7e cell culture

The cell line MO7e (a kind gift by Dr Asim Khwaja, Department of Haematology, University College London Medical School) was used as a positive control for the marker CD117 (Linnekin et al., 1995). The human myeloid leukaemia cell line was cultured in suspension with RPMI-1640 media (Sigma cat noR8758 supplemented with 10% FCS, Penicillin/Streptomycin and 10ng/ml GM-CSF [R&D systems, cat no215GM005]) in a humidified incubator at 37°C, 5% CO_2. The cells were diluted every 3-4 days to a concentration of $3x10^5$ cells/ml in fresh media.

2.4.1.2 Weri-Rb-1 cell culture

The human retinoblastoma cell line Weri-Rb-1 (ATCC cat noHTB-169) suspension culture was used for the marker CD133 (Yin et al., 1997). The cells were cultured in RPMI media (Sigma 1/3 cat noR5886 and 2/3 cat noR8758 [to achieve a 10mM HEPES concentration] supplemented with 10% FCS, Penicillin/Streptomycin, fungizone, glucose (2.5g/L), glutamine (0.112g/L) and sodium pyruvate (1mM)) in a humidified incubator at 37°C, 5% CO_2. The media was changed every 3-4 days by centrifuging the cells at 125g for 4min and resuspending the cell pellet in fresh media. For subculture the cells were resuspended at a concentration of 2–3 $x10^5$ cells/ml in fresh media.

2.4.1.3 HepG2 cell culture

For hepatocyte markers, HepG2 (ECACC, Wiltshire UK) cells were used. HepG2s were cultured as an adherent monolayer in fully supplemented alpha-MEM media (as for PM excluding ferrous sulphate, nicotinamide and zinc sulphate and with no additional glucose [1000mg/L glucose is already present in media]) in a humid incubator at 37°C, 5% CO_2. The media was changed every 48hrs. For subculture the cells were trypsinized at 70-80% confluency. Centrifugation was at 300g for 4min.

2.4.1.4 HT29 cell culture

HT29 cells were used as a positive control for markers ABCG2 and HEA125. The cells were grown in DMEM (GibcoBRL cat no32430–027) supplemented with 10% FCS, Penicillin/Streptomycin, fungizone and glucose (2.5g/L) in a humid incubator at 37°C, 5% CO_2. The media was changed every 3-4days. For subculture the cells were trypsinized at 70-80% confluency. Centrifugation was at 300g for 5min.

2.4.1.5 CEM/VLB cell culture

The multidrug resistant human leukemic cell line CEM/VLB (vinblastine resistant) cells were used as a positive control for P-glycoprotein expression (Pgp) (Beck et al., 1988). Cells were provided by Dr Ganeshaguru Kanagasabai, Department of Haematology, Royal Free Hospital. In brief, cells were cultured in DMEM culture media supplemented with 10% FCS.

2.4.2 Other positive controls

2.4.2.1 Human peripheral blood monocytes (PBMCs)

20ml of EGTA treated human peripheral blood was lymphoprepped and washed twice before being used as a positive control for markers CD34, CD45 and CD49f.

2.4.2.2 Human liver tissue

Tissue from human liver explants and resected tissue was collected for immunohistochemistry (formalin-fixed paraffin embedded blocks 2.6.3) and mRNA analysis (snap frozen in liquid nitrogen 2.5).

2.4.3 IgG controls

IgG controls raised in the same host towards an unrelated antigen were used to investigate non-specific binding of immunocyto/histological and flow cytometric antibodies.

2.5 Gene expression by RT- PCR

2.5.1 Cell lysis

mRNA was isolated using the cell-to-cDNA kit II (Ambion cat no1722/3). The cells were washed once with PBS to remove any remaining FCS and incubated for 10min with lysis buffer at 75°C. The lysis buffer breaks the cells to expose the RNA and also deactivates any RNases present. DNase I was added to the cell lysate and incubated for 15min at 37°C to degrade the genomic DNA. The DNase I was then heat inactivated by heating the samples at 75°C for 5min.

2.5.2 cDNA preparation

cDNA was generated by reverse transcription using the cell-to-cDNA kit II (Ambion cat no1722/3). Samples were incubated with a dNTP mix and random decamers for 3min at 70°C. RT buffer, M-MLV reverse transcriptase and RNase inhibitor was added and the samples incubated for 15-60min at 42°C. The samples were kept at 92-95°C for 10min to inactivate the reverse transcriptase. –RT controls (containing water instead of the M-MLV enzyme) of all samples were conducted alongside the experiments to detect any genomic DNA present. The samples were kept at -20°C for future use.

2.5.3 Polymerase Chain Reaction (PCR)

PCR was performed using HotStarTaq Master Mix Kit (Qiagen cat no 203443/5) containing dH_2O and HotStarTaq Master Mix with HotStarTaq DNA Polymerase, PCR buffer and dNTPs. The PCR reactions contained template cDNA (1μl), forward and reverse primers (1μl each of 50μg/ml stock [Sigma-genosys]), dH_2O (7μl) and HotStarTaq Master Mix (10μl, i.e. used at a ratio of 1:1 of the total volume of 20μl). A template negative control was used for all reactions.

The samples were placed in a thermal cycler (Applied Biosystems GeneAmp PCRSystem 2700) with the following protocol for 40 cycles.

Hot start: 95°C / 15min

DNA denaturation/melting: 94°C / 30s

DNA-primer annealing: 58-68°C / 30s

Primer extension: 72°C / 30s-1min20s

Hold: 72°C / 10min

Hold: 4°C

The hot start is required to activate the DNA polymerase. DNA-primer annealing temperature is dependent on the specific primers used and is dependent on GC content of the primers. The primer extension time is dependent on the size of the template sequence; longer fragments require longer extension times.

2.5.4 Primer sequences, annealing temperatures and elongation times

The primers used for PCR reactions are listed in Table 2-1.

Table 2-1 The primers used for PCR reactions are listed with primer sequences, size of PCR product, together with Annealing (Ann.) temperature and elongation time (Elong.).

Target	Forward	Reverse	Size (bp)	Ann.	Elong.
GAPDH	TGAAGGTCGGAGTCAACGGATTTGGT	CATGTGGGCCATGAGGTCCACCAC	983	68°	1'20''
B-actin	TGGCACCACACCTTCTACAATGAGC	CATGTGGGCCATGAGGTCCACCAC	983	68°	1'
Albumin	CCTTTGGCACAATGAAGTGGGTAACC	CAGCAGTCAGCCATTTCACCAT	355	58°	1'20''
C-met	AGAAATTCATCAGGCTGTGAAGCGCG	TTCCTCCGATCGCACACATTTGTCG	441	58°	1'20''
AAT	AGACCCTTTGAAGTCAAGGACACCG	CCATTGCTGAAGACCTTAGTGAT	359	68°	1'
GGT	GACGACTTCAGCTCTCCCAG	CTTGTCCCTGGATTGCTTGT	489	62°	2'
Cyp1B1	GAGAACGTACCGGCCACTATCACT	GTTAGGCCACTTCAGTGGGTCATGAT	357	62°	2'
Oct-4	CGRGAAGCTGGAGAAGGAGAAGCTG	CAAGGGCCGCAGCTTACACATGTTC	241	58°	1'20"
AFP	AGAACCTGTCACAAGCTGTG	GACAGCAAGCTGAGGATGTC	676	58°	1'20"
BGP	ATGGAACATTCCAGCAAAGC	GGAGTGGTCCTGAGTGTGGT	428	60°	45''
EGF-R	CAGTCGTCAGCCTGAACATAACATCC	AGGTTGCACTTGTCCACGCATTCCC	307	68°	1'20''
TGFβ-R	TAGTCACTGACAACAACGGTGCAGTC	ACAGTGCTCGCTGAACTCCATGAGC	539	68°	1'20''
CK18	GAGATCGAGGCTCTCAAGGA	CAAGCTGGCCTTCAGATTTC	357	58°	1'
CK19	CTACAGCCACTACTACACGAC	CAGAGCCTGTTCCGTCTCAAA	148	52°	1'
CK7	CGTGCGCTCTGCCTATGG	GCGGTTAATTTCATCTTCGT	409	52°	1'
ABCG2	GGGTTCTCTTCTTCCTGACGACC	TGGTTGTGAGATTGACCAACAGACC	373	62°	1'
CD117	GAGTTGGCCCTAGACTTAGA	CCTGGAGTTGGATGCAAGTT	749	64°	1'
hTERT	TCCATCAGAGCCAGTCTCACCTT	GTCCAGGATGGTCTTGAAGTCTG	540	62°	45''
18s	GTATTGCGCCGCTAGAGGTG	CTGAACGCCACTTGTCCCTC	524	50°	30''

2.5.5 Agarose gel electropheresis

1-2% agarose powder (Invitrogen cat no 15510-027) was dissolved into 50-100ml 1xTAE (0.04M Tris-acetate, 0.001M EDTA) by heating for 2-3min in a microwave. The solution was allowed to cool down to around 40°C and ethidium bromide (Sigma cat no E8751) was added (final concentration 0.2μg/ml). The solution was poured into a gel cast and allowed to gel.

5μl PCR product was mixed with 2μl DNA loading buffer (Bioline cat no BIO-37045) and 5μl of the mixture was loaded into the wells. 5μl of an appropriate DNA Hyperladder (Bioline cat no BIO-33026 [I] or HTPL-4200 [IV]) was used on each gel. Electrophoresis was carried out at constant voltage 90V/140mA in 1xTAE buffer (0.04M Tris acetate, 0.001M EDTA, pH 8.0) for 45min. The bands were visualised under UV light and photographed.

2.6 Immunocyto/histochemistry

2.6.1 Cytospins

Cells were prepared as a single cell suspension. $2x10^4$ – $1x10^5$ cells in a volume of 0.1-0.5ml were loaded into double cytofunnels (ThermoShandon cat no1102547) and centrifuged onto glass slides with a cytospin (ThermoShandon Cytospin4). The cells were spun with medium acceleration for 4min at 600-1000rpm depending on the cell type.

1. Non-parenchymal cells: 1000rpm
2. HepG2/Primary Hepatocytes: 700rpm
3. HS-5: 600rpm
4. WERI-Rb-1: 600rpm
5. MO7e: 600rpm
6. Lymphocytes: 800rpm

The slides were then air-dried for 15-30min, wrapped in clingfilm and stored at -20°C for future use.

Cytospin slides were allowed to reach room temperature before unwrapping. The slides were fixed, depending on the antibody, by formalin for 4min at room temperature or

10min ice-cold 80% acetone or 10min ice-cold methanol. The slides were air dried and rehydrated with Tris-buffered saline (TBS – 25mM Tris, pH 7.4) for 5min. The slides were then ready for EnVision staining.

2.6.2 *In-situ* wells

Cell culture wells were washed twice with medium to remove cell debris. The cells were then fixed depending on the primary antibody used (as for cytospins) and rehydrated with TBS for 5min. The slides were then ready for EnVision staining.

2.6.3 Formalin Fixed Paraffin Embedded (FFPE) sections

Tissue was fixed in formalin for 12-60hrs and processed with routine histopathology samples. The tissue was mounted in paraffin blocks. The sections were cut (4μm) and mounted on APES-coated slides and left to dry for at least 12hrs. The sections were de-paraffinised with xylene (3x3min) and rehydrated by passing through 100% (1min) and 70% (1min) alcohol into water (5min). Slides were pre-treated if necessary, depending on the primary antibody.

2.6.3.1 Antigen unmasking: Heat pre-treatment

The slides were immersed in citrate saline buffer (0.01M citrate, NaCl 0.15M, pH 6.0) and microwaved on full power for 5min. The reaction was stopped by immersing the slides in cold water.

2.6.3.2 Antigen unmasking: Trypsin digestion

The slides were covered with pre-warmed trypsin solution (0.1% [0.1g in 100ml] trypsin [ICN Biomedical, cat no 150215] in 0.05M Tris/HCl, 0.15M NaCl pH7.8) at 37°C for proteolytic digestion and incubated in a humid incubator at 37°C for 30min. The reaction was stopped by rinsing the slides with distilled water.

2.6.4 Staining with EnVision

The EnVision system (DAKO cat no K5007) is based on an HRP labelled polymer which is conjugated with secondary antibodies specific for mouse and rabbit primary antibodies. The labelled polymer does not contain avidin or biotin, therefore eliminating non-specific staining resulting from endogenous biotin activity in liver samples.

The samples were prepared appropriately and then blocked for endogenous hydrogen peroxide by peroxidase-blocking solution (DAKO cat no S2023) for 5min and washed with distilled water. The samples were blocked for 15min with goat serum (DAKO, cat noX0907) 1:10 in TBS to remove non-specific binding of the secondary antibody. The primary antibody was incubated for 60min at room temperature and the samples washed twice with TBS for 5min. The secondary antibody was incubated for 30min at room temperature and samples washed three times with TBS for 5min. The DAB+ Chromogen (3,3'-diaminobenzidine chromogen solution) was added for 5-10min and rinsed off with distilled water. The samples were counterstained with haematoxylin for 5min (sections and cytospins) - 15min (cells in culture), washed in water, dipped in acid alcohol (5s) and washed in water. The slides were then mounted on coverslips by dehydrating the slides and using DPX mounting media.

2.6.4.1 Primary antibodies

Table 2-2 Antibodies used for immunocyto/histochemistry.

Antigen	Clone	Cat no	Conc µg/ml	Dilution	Fixing	Pre-treatment
HEA125	HEA125	Autogen Bioclear AB172	50	1:50	Formalin	Trypsin
CK19	b170	Novocastra NCL-CK19	19	1:100	Formalin, Acetone	Trypsin
CD117	polyclonal	Dako A4502	200	1:50	Acetone, Formalin	Heat 5min
CD133	293C3	Miltenyi Biotech AC133	50	1:20	Acetone, Methanol	-
c-met	c-28	Santa Cruz Sc-161	200	1:100	Methanol, Acetone	-
c-met	h-190	Santa Cruz Sc-8307	200	1:10		-
CK8	TS-1	Novocastra NCL-CK8-TS1	32	1:100	Acetone	-
CK18	DC-10	Novocastra NCL-CK18	100	1:20	Acetone	-
Fibroblast	5B5	Dako M0877	155	1:50	Acetone	-
CK7	OV-TL 12/30	Dako M7018	260	1:50	Acetone	Trypsin, Heat 5min
A-SMA	1A4	Sigma A2547	2200	1:400	Formalin	no
HSA	OCH1E5	Dako M7516	17	1:50	-	no
CD68	PG-M1	Dako H7122	125	1:50	Acetone	no
CD34	QBEND/10	Dako N1632	50	1:25	Acetone	Heat 5min
Mouse IgG		Sigma I8765	10 500			
Rabbit IgG		Sigma I8140	11- 11 500			

2.6.4.2 Negative controls for immunohisto/cytochemistry

Non-specific mouse or rabbit IgG controls were used as negative controls for primary antibodies. Both the species and specific IgG concentrations were taken into account.

2.6.5 Haematoxylin and Eosin (H&E) staining for histology

For histological examination of slides, cytospins and cells in culture, the samples were H&E stained. Haematoxylin stains the nuclei blue, whereas eosin stains the cytoplasm pink. The samples were immersed in haematoxylin for 5-15min, washed in water, dipped into acid alcohol and left in water for 5min. The samples were then immersed in eosin for 3-5min and then washed in water before dehydrating and mounting the slides with DPX.

2.7 Enzyme Linked Immunoadsorbent Assay (ELISA)

The ELISA was used to measure liver specific proteins secreted into the culture media. The ELISA method uses a pre-coated capture antibody in a 96-well plate, which binds to any antigen present in the conditioned culture media which is placed in the wells. The antibody-antigen complex was then observed with a horseradish peroxidase (HRP)-conjugated secondary antibody. The HRP-labelled complexes are observed when hydrogen peroxidase (H_2O_2) is added and the cleavage of the hydrogen peroxidase to the oxidation of a hydrogen donor which changes colour during the reaction. The amount of albumin and AAT was observed in the conditioned culture media.

The capture antibodies (rabbit anti-human albumin [Dako cat no A0001 (1/1400)] and rabbit a-AAT [Dako cat no A0012 (1/900)]) were diluted in coating buffer (0.318g sodium carbonate and 0.586g sodium hydrogen carbonate in 200ml distilled water, pH 9.2). Albumin standards were Human serum albumin (Sigma cat no A-3782) with the range of 25-200ng/ml. AAT standards were Citrated human plasma with the range of 12.5-200ng AAT/ml. The capture antibodies were plated onto 96-well plates (Invitrogen cat no 442404) at 100µl/well, wrapped in cling-film and left for 2hrs at 37°C or overnight at 4°C.

Plates were washed three times with wash buffer (PBS with 0.05% Tween 20) and the standards and samples were plated at 100µl/well in triplicate. The plates were wrapped in cling-film and incubated at 37°C for one hour. The plate was washed three times with wash buffer using an automated plate-washer. The secondary HRP-conjugated antibody (HRP conjugated rabbit a-human albumin [Dako cat no P356 (1/8000)] or HRP conjugated rabbit a-human AAT [Dako PE876 (1/1000)]) diluted in wash buffer was added to the plate at 100µl/well, wrapped in cling-film and incubated at 37°C for one

hour. The plate was washed five times with washing buffer. 100µl/well of OPD (2 tablets OPD (Dako cat no S2045) dissolved in 12ml distilled water with 6µl 30% hydrogen peroxide added immediately prior to use) was added at timed intervals and kept protected from light until a sufficient colour change was observed. The reaction was stopped with 50µl/well sulphuric acid (2M). An ELISA plate reader was used to read the absorbance at 492nm (Anthos HTII).

2.8 Telomerase activity –TRAP assay

The telomeric repeat amplification protocol (TRAP assay) was carried out using TeloTAGGG Telomerase PCR ELISA Plus kit (Roche cat no2013789). The TRAP assay is a photometric enzyme immunoassay for quantitative determination of telomerase activity.

Telomerase activity was measured in approximately $2.8x10^4$ non-parenchymal cells from culture (npcRTx). The immortalised cell line HepG2 cells were used as a positive control (Suda et al., 1998) and heat inactivated cells as a negative control. The kit method is optimised for starting with $2x10^5$ cells, but only 1000 – 3000 cells are used for the telomeric repeat amplification step per sample. HepG2 controls were set in the range of the non-parenchymal cell density tested.

The cells were trypsinized and washed with PBS and resuspended in lysis reagent. Samples with $2x10^5$ cells were resuspended in 200µl, whereas samples with approximately $2.8x10^4$ were resuspended in 20µl. The samples were incubated for 30min on ice. The lysates were centrifuged at 16 000g for 20min, and the supernatant was transferred to a fresh tube. The centrifugation step was repeated and samples were then snap frozen in liquid nitrogen and stored at -80°C.

Heat inactivated controls were heated to 85°C for 10min. 25µl reaction mixture (containing biotinylated telomerase substrate, optimized anchor primer, nucleotides and Taq DNA polymerase) for a one-step telomerase-mediated primer elongation and PCR amplification, and 5µl internal standard (used as an internal amplification control to detect any inhibitors) were mixed with 3µl of each sample. 3µl corresponds to approximately 4.2×10^3 cells for samples containing $2.8x10^4$. A positive control with 1µl control template was set up, and a negative control with 1µl lysis reagent. Nuclease-

free water was added to a total volume of 50µl. Samples were placed in a thermal cycler (details in Table 2-3). The resultant samples were stored at -20°C.

Table 2-3 PCR amplification details for the TRAP assay.Thirty cycles of amplification, denaturation, annealing and polymerisation was performed.

PCR cycles	Elongation	Telomerase inactivation	Amplification	Denaturation	Annealing	Polymerisation	Hold
30	25° 20'	94° 5'	94° 30"	50° 30"	72° 1' 30"	72° 10'	4°

For ELISA detection, 2.5µl amplification product was incubated at room temperature for 10min with 10µl denaturation reagent. 100µl Hybridisation buffer T (containing DIG-labeled detection probe complementary to telomeric repeat sequence) and 100µl Hybridisation buffer IS (containing DIG-labelled detection probe complementary to internal standard) was added for each sample (two separate tubes) and vortexed. The solutions were transferred to the pre-coated MTP modules supplied with the kit and the wells were covered with self-adhesive foil. The MTP modules were incubated at 37°C on an elliptical shaker for 2h. The hybridisation solution was removed and the wells washed three times with washing buffer (250µl). Anti-DIG-HRP working solution was added, wells covered with foil and incubated at room temperature for 30min while the plate was rotating. The solution was removed and modules washed five times with washing buffer (250µl). TMB substrate was added, wells were covered with foil and incubated for colour development at RT for 10-20min while the plate was rotating. Without removing the reacted substrate stop reagent was added to stop colour development. A microtiter plate reader was used to measure absorbance of samples at 450nm within 30min after adding stop reagent. A reference wavelength of 690nm was not used due to the microtiter plate reader used.

The assay contained controls for the assay as well as for the sample. The negative control for the assay contained cell lysis reagent, instead of the sample containing cells. The positive control template contained a positive control template DNA with the same sequence as a telomerase product with 8 telomeric repeats [Low = 0.001 amol/µl] (provided by the kit). Each sample had a heat inactivated negative control and an internal standard. The internal standard aimed to test for any inhibition of the amplification process by producing a different PCR segment (216bp) that would be identified by the assay if the amplification worked.

The TRAP-assay kit recommended a method for analysing the samples: "Subtract the mean of the absorbance readings of the negative controls from absorbance readings of the samples. Samples are to be considered as telomerase-positive if the difference in absorbance is higher then the twofold background activity" (Roche, cat no 2 013 789). As a reference wavelength (690nm) could not be used, the quantification of the level of activity was not applicable, and the results are only expressed as negative or positive for telomerase activity. The calculation presented here shows the comparison between the NpcRTx sample and the HepG2 control.

The negative controls, including the heat treated samples, were averaged (1). The value was multiplied by two (2) and compared with the sample value from which the mean of the negatives had been subtracted (3). If the sample value after the subtraction was higher than twice the mean, the sample had telomerase activity (4).

1) Negative controls averaged: (0.091+0.078+0.065)/3 = 0.078
2) The double of the mean of negatives 2x0.078 = 0.156
3) NpcRTx sample value: 0.071, Mean of negatives: 0.078
   0.071-0.078 = -0.007
   HepG2 (28 000 cells) sample value: 2.553, Mean of negatives: 0.078
   2.553-0.078 = 2.475
4) NpcRTx sample value after subtraction: -0.007
   HepG2 (28 000 cells) sample value after subtraction: 2.475
   Double of the mean: 0.156
   NpcRTx: -0.007 < 0.156 – telomerase negative
   HepG2 (28 000 cells): 2.475 > 0.156 – telomerase positive

2.9 Transduction with hTERT

2.9.1 Producing virus particles – calcium phosphate transfection

293gp cells [Clontech BD] were cultured in 10cm petridishes (DMEM supplemented with 10%FCS, antibiotics and fungizone) to confluency. The cells were passaged 1:2 the day before use. For use, the cells need to be 75% confluent and the media was changed 1-2hrs before transfection.

20μg vector DNA pBabepuro-EST2 for hTERT or pHMIV-eGFP for GFP control was mixed with 5μg VSVG plasmid DNA encoding the virus enevelope proteins. The solution was made up to 450μl with water and 150μl of 2.5M $CaCl_2$ was added. The solution was mixed by vigorously bubbling air through the mixture using a Pasteur pipette. While bubbling, 500μl 2xHBS (50mM HEPES, pH 7.0, 1.5mM Na_2HPO_4, 280mM NaCl) solution was added dropwise. The solution was kept stationary for 20-30min at room temperature for the DNA to precipitate. The DNA mixture was added to the 293gp cells dropwise by rocking the plate side to side to allow even distribution and to avoid the toxic effects of large amounts of DNA. The cells were left overnight in a 37°C/5% CO_2 humid incubator. The GFP controls were observed under a fluorescent microscope to determine if the transfection had worked. The media was then removed, cells washed with PBS and replaced with fresh media. The plates were left for three days to collect viral particles.

2.9.2 Transducing non-parenchymal cells

After collection, the viral supernatant was filtered through a 0.45μ filter to remove any remaining 293gp cells. The viral supernatant was mixed in a ratio of 1 to 1 with PM culture media used in normal culture for the non-parenchymal cells. The remaining virus was frozen at -80°C. Polybrene was added to the virus supernatant/PM mixture at a concentration of 8μg/ml (Polybrene/Hexadimethrine bromide, Aldrich cat no10, 768-9). The non-parenchymal cells were incubated for 8hrs with the viral particles and the media was then replaced with fresh PM media. After six days, the transduction was repeated with the frozen virus particles. The hTERT expressing cells were selected with puromycin (Sigma, cat noP-7255) on two different occasions (day 63 and 151) after transduction. Puromycin was added to the culture media for 7 days at 1μg/ml.

2.10 Staining non-parenchymal cells for flow cytometric analysis, sorting and magnetic cell sorting

Thawed non-parenchymal cells were resuspended in a small volume of buffer and incubated with the primary antibody/antibodies. The amount of antibody, as well as the labelling volume, time and temperature are found in Table 2-4 for all antibodies used for flow cytometry and sorting, and MACS antibodies are found in Table 2-5. The cells were washed in 10-20 times the labelling volume of buffer, and recovered by

centrifugation at 200g for 10min. For flow cytometry, if the primary antibodies were not directly conjugated to a fluorochrome a fluorochrome-linked secondary antibody was used. The cells were then washed and resuspended at 250-400µl/10^6 cells for flow cytometry. For flow cytometric analysis $1x10^5 - 10^6$ cells/sample were used.

2.10.1 Control antibodies

Isotype-matched controls were used to confirm low non-specific binding of the antibodies used. They are listed in Table 2-4.

Table 2-4 Antibodies used for flow cytometry. Amount of antibody (µl ab/sample), labelling volume (volume), incubation time (time) and temperature (temp). RT = room temperature indicated.

Antigen	Clone	Cat no	µl ab/sample	Volume (µl)	Time (min)	temp
CD117-Cy5	95C3	Beckham Coulter PNIM2657	10	250	15	RT
CD133/1-PE	AC133	Miltenyi Biotech 130-080-801	0.25	250	15	RT
CD45-FITC	J33	Beckham Coulter PNIM0782	2.5	250	15	RT
CD34-APC	581	Beckham Coulter PNIM2472	2.5	250	15	RT
c-met	DL-21	Upstate 05-238	5	250	15	RT
c-met	DO-24	Upstate 05-237	5	250	30	4-8°C
c-met	poly	R&D Systems AF276	5	250	15	RT
Control c-met	-	R&D Systems AB-108-C	5	250	15	RT
CD49f	MP4F10	R&D Systems MAB1350	2	100	15	RT
Control CD49f	-	R&D Systems MAB0041	2	100	15	RT
ABCG2-PE	5D3	R&D Systems FAB995P	10	25	45	4-8°C
Control ABCG2-PE	-	R&D Systems IC0041P	10	25	45	4-8°C
HEA125	HEA125	Autogen Bioclear AB172	1	100	15	RT
goat IgG - Alexa488	poly	Molecular Probes A-11055	0.5	250	15	RT
goat IgG - PE	poly	ImmunoResearch 705-116-147	2	200	15	RT
mouse IgG2B-PE	poly	BD Biosciences 340269	20	-	15	RT
mouse IgG - FITC	poly	BD Biosciences 554001	1	100	15	RT

Table 2-5 Antibodies used for MACS. Amount of antibody (µl ab/sample), labelling volume (volume), incubation time (time) and temperature (temp). RT = room temperature indicated.

Antigen	Clone	Cat no	µl ab/sample	Volume (µl)	Time (min)	temp
CD117 - bead	AC126	Miltenyi Biotech 130-091-332	100	400	15	RT
CD117/2 – PE	A3C6E2	Miltenyi Biotech 120-002-135	10	500	5	RT
CD133 - bead	AC133	Miltenyi Biotech 130-050-801	100	400	30	RT
CD133/2 - PE	293C3	Miltenyi Biotech 130-090-853	20	500	10	RT
CD34 - bead	QBEND/10	Miltenyi Biotech 130-091-586	50	450	15	4-8°C
CD34/2 - PE	AC136	Miltenyi Biotech 130-091-586	50	500	5	4-8°C
c-met	poly	R&D Systems AF276	50	2500	15	RT
goat IgG - PE	poly	ImmunoResearch 705-116-147	20	2000	15	RT
PE - bead	poly	Miltenyi Biotech 130-048-801	200	800	15	RT

2.11 Functional calcein-AM assay

The calcein-AM assay studying P-glycoprotein function was optimised and performed with Dr Ganeshaguru and Rob Baker, Department of Haematology, Royal Free Hospital.

$0.5x10^6$ cells/sample were resuspended in 1ml DMEM (+ 10% FCS). The cells were pre-incubated with inhibitors PSC833 (10µl of 10µM stock) and/or fumitremorgin C

[FTC] (10μl of 0.05μM stock) for 5min at 37°C. Calcein-AM was added (10μl of 100μM stock [Sigma cat no148504-34-1]) 15min 37°C. The cells were then washed in PBS. For each sample, four conditions were set-up:

1) calcein-AM only

2) calcein-AM + P-glycoprotein blocker PSC833

3) calcein-AM + ABCG2 blocker FTC

4) calcein-AM + both blockers

The cells were analysed for fluorescent calcein by flow cytometric analysis in the FL-1 channel.

2.12 Additional methods

Methods specific to the different chapters are found at the beginning of each chapter. Below is a list of the specific methods used.

Chapter 3

- In-situ immunocytochemistry
- Irradiated HS-5 feeder cell layers

Chapter 4

- TRAP assay
- hTERT transduction
- Differentiation studies using growth factors

Chapter 5

- 1 colour flow cytometric analysis
- 2 colour flow cytometric analysis and compensation
- 4 colour flow cytometric analysis
- Calcein-AM functional assay

Chapter 6

- Flow cytometric cell sorting
- Magnetic cell sorting
- Methylcellulose culture
- Liver supernatant production from liver tissue

Chapter 3

Non-parenchymal cells in culture

3.1 Introduction

3.1.1 Identifying a stem cell

The definition of a stem cell is "A cell that can give rise to a lineage of cells. A cell that upon division produces dissimilar daughters, one replacing the original stem cell, the other differentiating further" (Medical Dictionary, 2003). A lot of research has been carried out in order to look for something specific to identify a cell that has potential to differentiate into different lineages (see *Chapter 1*). Surface markers (CD34, CD133 and CD117), transcription factors (Oct-4) and growth potential under specific growth conditions (growth factors, matrices) have been used to enrich for stem cells.

This chapter aimed to take a step backwards and look at a mixed population of non-parenchymal cells in culture. Specific parameters were not laid out, but the potential and behaviour of the different cells were assessed. Different culture conditions and explant livers of multiple disease aetiologies were used. The proliferation, morphology, cell surface markers and mRNA and protein expression patterns were observed. The possibilities with this approach included the isolation of potential stem/progenitor cell populations which would have a new and unique combination of characteristics.

3.1.2 Culture of putative progenitor cells

There are two important papers regarding cell isolation from human explant livers for subsequent culture. In our laboratory, Selden *et al.* were able to isolate cells expressing hepatocyte, biliary epithelial and stem cell phenotypic markers from an explant liver from a patient with sub-acute hepatic failure (Selden et al., 2003). Crosby *et al.* (Crosby et al., 2001; Crosby et al., 2002), on the other hand, were able to isolate stem-like cells from human adult liver which acquired biliary epithelial cell markers in culture. Stem cell factor receptor CD117 (c-kit) or the haematopoietic stem cell marker CD34 were used to pull out the cells. The cells were isolated from human diseased liver (primary biliary cirrhosis and alcohol liver disease), as well as normal human liver, although in smaller numbers.

The main difference between the two studies is the culture period. Cells isolated by Selden *et al.* were cultured long-term, i.e. for greater than 165 days. The cells secreted albumin and alpha-1-antitrypsin protein and RT-PCR showed expression of a hepatocyte phenotype (CK18, albumin and alpha-1-antitrypsin), biliary epithelial phenotype (CK7 and CK19, biliary glycoprotein) and markers common on both cell types (c-met, TGF-beta-receptor II and gamma-glutamyl-transpeptidase). The cells also expressed the stem cell transcription factor Oct-4 (Selden et al., 2003). Together this data suggests that the cells were bipotential, as they were expressing markers of both cell types.

Cells derived by Crosby *et al.* were cultured short-term, i.e. for seven days. The cells were kept in biliary growth medium, and two distinct morphologies arose. The isolated cells expressed either CK19 (biliary epithelial cell marker) or CD31 (endothelial cell marker) after seven days in culture. However, even though the cells were pulled out by using markers CD117 and CD34, the expression of these markers was lost in culture. The isolated cells did not express CK19 or Human Epithelial Antigen 125 (HEA125) 24hrs after isolation, suggesting that the cells have differentiated by losing some markers and gaining others (Crosby et al., 2001).

The origin of the cells isolated in the two cases is uncertain. These cells could be adult stem cells or liver progenitors residing in the liver, activated only during severe damage to the liver and when hepatocytes are unable to proliferate. However, bone marrow cells have also been known to migrate and differentiate into mature cells in the liver (see *Chapter 1*). Whichever scenario is true, diseased liver is likely to be enriched in activated stem cells or liver progenitors.

The cells isolated by Selden *et al.* were a mixture of non-parenchymal cells, whereas Crosby *et al.* used markers to specifically investigate a certain subpopulation of cells. As no definite stem cell marker for liver progenitors/stem cells has been found, it is reasonable to take a step backwards to investigate all non-parenchymal cells as putative progenitor cells.

Human explant livers from a variety of disease aetiologies (primary biliary cirrhosis, secondary biliary cirrhosis, alcohol liver disease, cryptogenic cirrhosis, acute and sub-acute fulminant liver failure) were available for investigation. The non-parenchymal cell

isolation by liver perfusion was already established in our laboratory (Selden et al., 2003).

3.2 Hypotheses

The hypotheses of this chapter were:

1) Human explant livers are enriched in liver stem cells/hepatocyte progenitors which can be isolated from the non-parenchymal cell fraction by liver perfusion.
2) The isolated progenitor cell populations can be grown long-term in culture because of the nature of stem cells.

3.3 Aims

The aims of this chapter were to:

1) Isolate viable non-parenchymal cells from liver explants by liver perfusion.
2) Establish long term culture.
3) Observe the growth patterns of a mixed population of non-parenchymal cells.
4) Investigate colonies of non-parenchymal cells as candidate stem cells by tracking their proliferation and expression of hepatocyte, biliary epithelial and stem cell markers.

3.4 Methods

3.4.1 Starting material – Non-parenchymal cell samples

The non-parenchymal cells were isolated from human explant livers and BS resected tissue as described in *Chapter 2 General Methods* using collagenase liver perfusion. Table 3-1 lists the liver explants used in this chapter.

Table 3-1 Non-parenchymal cell samples cultured.

Disease aetiology	Number of samples (n)
ALD	4
PBC	5
PSC	2
Cryptogenic	2
Sub-fulminant	1
Fulminant	2
Re-transplant	2
2ndry BC	1
BS resection	3

The cells were cultured in PM, PM supplemented with growth factors (HGF, EGF, FGF-4, PDGF-BB and GCSF) [PM/GFs] and 1:1 mixture of PM and HS-5 conditioned media (PM/HS-5) as well as on irradiated HS-5 feeder cells. For preparation of feeder cells please refer to *Chapter 2 General Methods*. Non-parenchymal cells were seeded at $1.5x10^5$ viable cells/well in 48-well tissue culture plates.

3.4.2 Positive controls

Positive controls were used for optimising and controls for immunocyto/histochemistry and RT-PCR.

3.4.2.1 Human liver tissue

Human liver tissue was used as a positive control for all liver markers, for more detailed methods please refer to *Chapter 2 General Methods*.

3.4.2.2 Weri-Rb-1

Weri-Rb-1 cells express CD133 and were used as a control for experiments assessing this antigen. For culture and subculture, please refer to *Chapter 2 General Methods*.

3.4.2.3 MO7e

MO7e cells express CD117 and were used as a control for experiments observing this antigen. For culture and subculture, please refer to *Chapter 2 General Methods*.

3.4.2.4 HepG2

HepG2 cells express c-met and were used as a control for experiments observing this antigen. For culture and subculture, please refer to *Chapter 2 General Methods*.

3.4.3 Negative controls

Non-specific IgG controls at the specific antibody concentrations were used to confirm low non-specific binding of the antibodies used for immunocyto/histochemistry.

3.4.4 Gene expression by Reverse Transcription PCR

Methods described in *Chapter 2 General Methods*.

3.4.5 Protein expression patterns by immuncyto/histochemistry

Methods described in *Chapter 2 General Methods*.

3.4.6 Enzyme-Linked ImmunoAbsorbent Assay (ELISA)

Alpha-1-antitrypsin and albumin ELISA as described in *Chapter 2 General Methods*.

3.5 Results

3.5.1 Typical cultures

Observing non-parenchymal cells in culture is not clear-cut. All wells in culture contain a mixture of different cell types with different morphologies. It is often very difficult to distinguish between one type of colony and another as there is no clear interface between the two. Cells also change morphology depending on the confluency of the well. It is impossible to distinguish if a dominating cell type has taken over and grown on top of another or if another colony has grown and changed shape. Observations were made from daily, to once in two weeks. Due to the sheer numbers of wells to be observed for unusual colonies strict observations of the typical cultures were not pursued further.

Without in-depth analysis, four apparently different morphologies were observed. The pictures shown as examples are from a PBC explant after four weeks of culture.

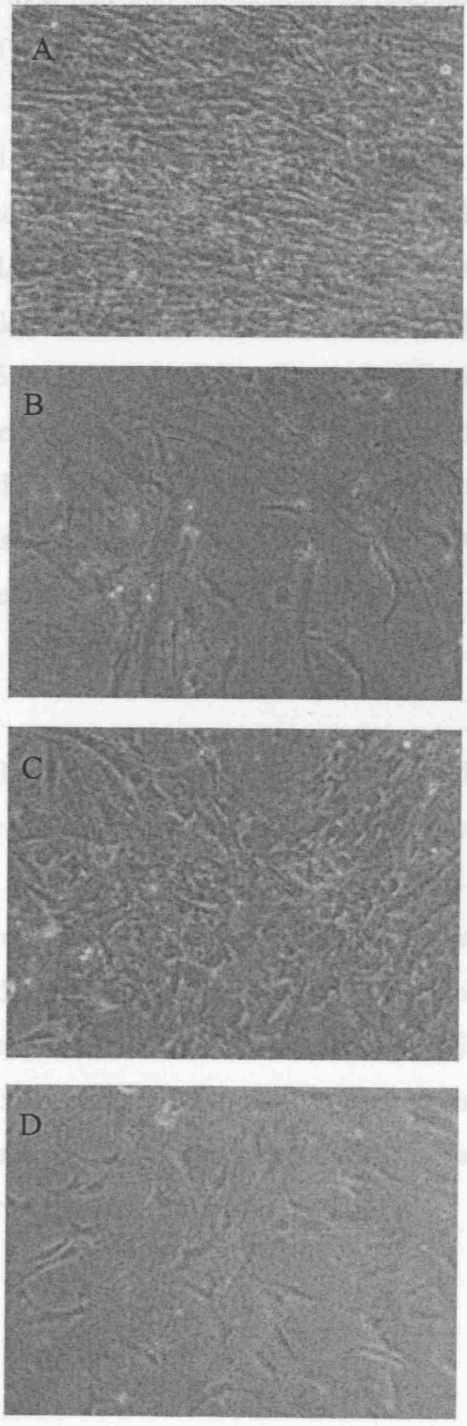


Fig. 3-1 Phase contrast microscopy x200 of non-parenchymal cell culture (PBC). Four types of morphology were identified. (A) Cells with a fibroblast-like morphology, (B) cells with a large cell morphology, (C) cells with criss-crossing fibroblast-like morphology and (D) cells with a star-shaped morphology.

The fibroblast-like cells were elongated and grew in the same direction. When confluent the cells grew on top of each other but still kept the directional growth. The large cells were spread out with wide protrusions to all directions. The cells grew in spirals around each other when confluent. The criss-crossing fibroblasts-like cells looked similar to the fibroblast-like cells, but they did not all grow in the same direction. The shape of the cells was not as consistent, even though they were all elongated. When confluent the cells grew on top of each other not respecting direction. The star-shaped cells had a relatively thick body surrounding the nucleus, but also had thin protrusions in random directions. When confluent, the cells grew on top of each other.

The PM, PM/GFs and PM/HS-5 cultures showed some differences. PM/GFs mostly contained rapidly growing (2-3weeks to reach confluency) fibroblast-like cells and criss-crossing fibroblast-like cells. The cells in PM/HS-5 contained the same cell types but were slower at reaching confluency (2-4 weeks). PM cultured cells contained all types of cells, and growing even more slowly (2-5 weeks to reach confluency). When trypsinised and reseeded all the cell types became similar to fibroblast-like cells and criss-crossing cells, suggesting that the cells could all be from a similar cell type but only with different growth patterns, or that some type of cell was better at surviving/recovering from the trypsinisation and therefore took over the culture.

3.5.2 Potential progenitor colonies

Anything deviating from the typical cultures was closely monitored. A stem cell should produce a proliferating colony, and should be a relatively rare event. Each well was observed closely and any cells growing in a defined colony, which were non-fibroblastic but with an epithelial morphology were noted [Fig. 3-2]. Epithelial-like colonies were seen in 0-4% of the wells cultured from any single explant liver. No particular disease aetiology was more enriched in potential colonies, but normal BS resections did not yield any colonies (n=3). The cell colonies were only present with other cell types in the same well. The epithelial-like morphology was similar to the cells isolated by Selden *et al.* and was used for further investigation (Selden et al., 2003).

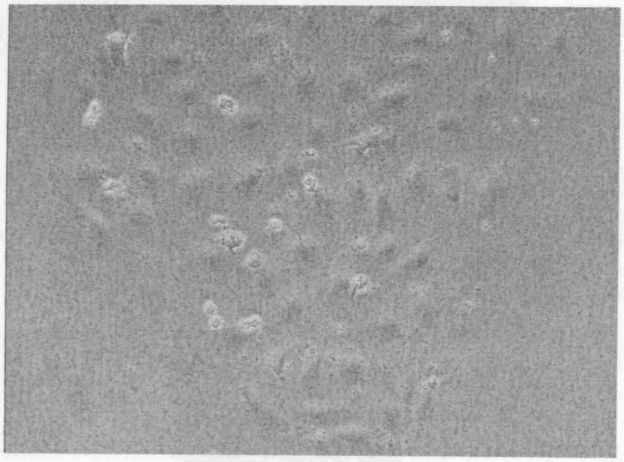


Fig. 3-2 Phase contrast microscopy x200. Cells with an epithelial-like morphology from non-parenchymal cells isolated from a PBC explant .

3.5.3 Immunocytochemistry of typical cultures and potential progenitor colonies

In-situ immunocytochemistry against a fibroblast marker, CK8 and CK19 was performed on a number of wells from a PBC explant Fig. 3-3. All types of cells stained with the fibroblast marker, even the cuboidal-like cells, suggesting this antibody was not very specific. No cell types stained for CK19 and only large cells with a lot of cytoplasm stained for CK8. The negative controls with the same amount of unspecific IgG were all negative (pictures not shown). All positive controls were positive, human primary hepatocytes for CK8 and FFPE human liver sections for CK19.

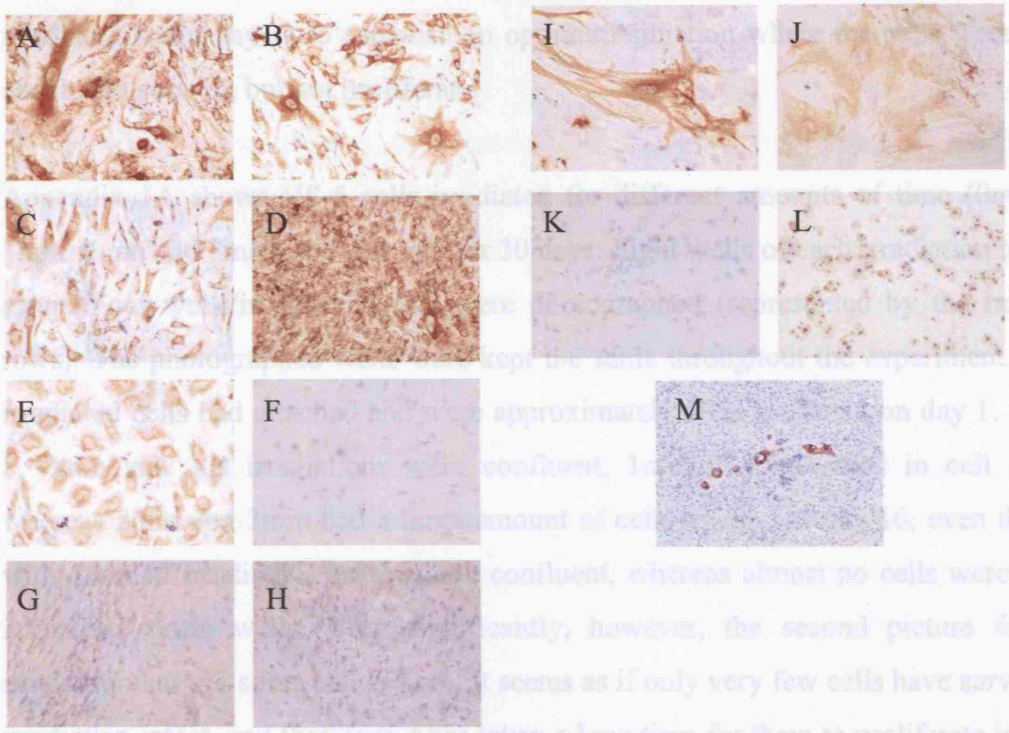


Fig. 3-3 In-situ immunocytochemistry on non-parenchymal cells in culture and immunohistochemistry on liver tissue. (A-E) Fibroblast marker stains all different types of cells. (F-H) The cells do not stain for CK19 and (I-K) only large cells stain for CK8. (L) Human hepatocyte cytospin as a positive control for CK8 and (M) human liver as positive control for CK19.

3.5.4 The feeder approach

Native and irradiated human HS-5 feeder layers were used to promote growth of progenitors. Many stem cells, for example haematopoietic stem cells and ES cells, prefer to be grown on a feeder layer. Even though it is possible to grow these cells on different matrices and with conditioned media from feeders, the best results are seen with a feeder layer, probably due to the cell-to-cell contact between the two cell types.

HS-5 cells are known to secrete a large number of cytokines and growth factors which are supportive of haematopoietic stem cell growth in vitro (Roecklein and Torok-Storb, 1995). Native and irradiated cells were plated in 48-well tissue culture plates and $1.5x10^5$ non-parenchymal cells were plated onto the feeders. HS-5 cells are a very rapidly growing fibroblast cell line. It usually takes 5 days for any of the non-parenchymal cells to attach, and by that time the native HS-5 cells had formed a sheet of

fibroblasts and were starting to lift off. Different irradiation times were used for the irradiated feeder layers to establish an optimum situation where the cells were able to attach and survive, but not proliferate.

Appendix 1A shows HS-5 cells irradiated for different amounts of time (0min, 30s, 1min, 2min and 3min) and cultured for 30 days. Eight wells of each irradiation time was setup. Four wells/irradiation time were photographed (represented by the horizontal rows). The photographed wells were kept the same throughout the experiment. All the irradiated cells had attached and were approximately 50% confluent on day 1. On day 8, 0min and 30s irradiations were confluent, 1min had increased in cell number, whereas 2min and 3min had a large amount of cells dying. On day 16, even the wells with 1min of irradiation had become confluent, whereas almost no cells were seen in 2min and 3min wells. Very significantly, however, the second picture for 2min irradiation has got some cell growth. It seems as if only very few cells have survived the irradiation intact, and therefore it has taken a long time for them to proliferate into large numbers. On day 23 this colony was even more obvious and another well at 2min also showed cell growth (not pictured). The cultures were terminated on day 30, at which point the well in 2min was confluent and one of the wells at 3min showed some growth.

These results were rather disappointing, because non-parenchymal growth on plastic cannot be seen before day 5, and by day 8 the 2 and 3min cultures are already dying. Moreover, the non-parenchymal cultures are often subcultured only after 3 weeks of culture, at which point the surviving HS-5 cells are already proliferating in 2min and 3min cultures. Any proliferating HS-5 cells will be of major importance if unidentified non-parenchymal cells are to be cultured.

A second experiment was set up with HS-5 irradiated cells to confirm the results (Appendix 1B). This time 0min, 1min30s and 4min were chosen as irradiation times. 20Gy has been quoted from the paper by Roecklein *et al.* (Roecklein and Torok-Storb, 1995) and equals to 4min in our gamma irradiator at a fixed dose. 1min30s was chosen as between the 1min, which became confluent on day 16, and the 2min, where most of the cells were dead on day16. On day 1 the cells are all attached and were 50% confluent. Day 6 the control 0min wells are confluent, whereas the cells in 1min30s and 4min wells are starting to detach. On day 15 the cell numbers in 1min30s has started to increase, whereas most cells have detached in the 4min wells. The cultures were

terminated on day 32 when the cultures in 1min30s were confluent and no surviving cells were observed in the 4min wells.

The feeder layer experiments were terminated because the cells that were irradiated for long enough to inhibit replication did not survive beyond day 6 in culture, which is required for non-parenchymal cell culture. Cells irradiated for a shorter time contained a proportion of cells able to divide, which would have interfered with any primary cell culture.

A pilot experiment was also conducted with non-parenchymal cells isolated from a PBC liver, where isolated non-parenchymal cells was seeded onto 20Gy irradiated and native HS-5 cells. The native cells overgrew into a sheet of fibroblasts and the irradiated cells died within a week. No interesting colonies were observed in either situation.

3.5.5 NpcRTx – A proliferating population of cells expressing a combination of hepatocyte, biliary epithelial and stem cell markers.

The npcRTx colony was identified in culture of cells isolated from a transplanted liver from a patient receiving a second transplant (after 21 days) because of ischaemia and rejection. The patient had received a course of GCSF therapy.

3.5.5.1 Markers expressed on the isolated cells before culture

Cytospins of the isolated fraction were stained by immunocytochemistry. Markers tested included CK8, CK18, c-met, CK19, CD117 and CD133 (Fig. 3-4). The background in freshly isolated primary cell cytospins was higher than for cell lines. This is probably due to impurities (cell debris, red blood cells etc.) present in the sample. It was therefore important to compare the staining to the negative IgG controls.

A number of cells were found to be expressing CK8 and CK18 (identified by arrows in Fig. 3-4 A and C). No such cells were seen in the corresponding IgG controls, even though there was high background for CK8 in some areas. The positive control primary human hepatocytes were positive. The cells staining were small in size, 10-20μm, but staining strongly. These cells could be a certain type of progenitor cell.

C-met expression was unclear, as there was no clear strong staining of the cells, but the overall staining was stronger than the IgG control. The positive control HepG2 cells were positive.

The non-parenchymal cells were negative for CK19, CD117 and CD133 when compared with the IgG controls with the corresponding antibody concentration. The positive controls were all positive. FFPE liver section was used as positive control for CK19, MO7e cells for CD117 and Weri-Rb-1 cells for CD133.

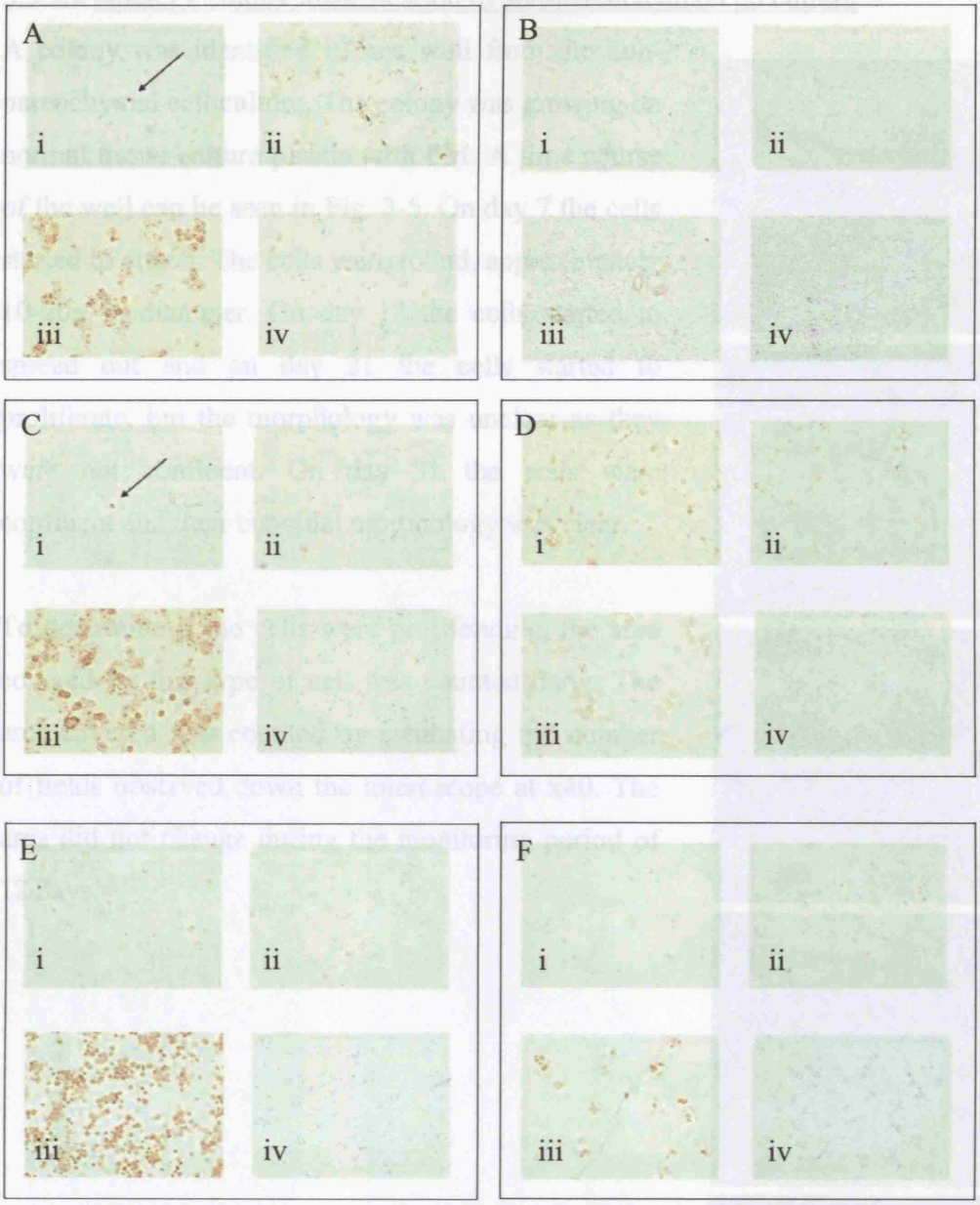


Fig. 3-4 Immunocytochemistry of non-parenchymal cell cytospins. Cells were isolated from a re-transplanted liver explant (the colony npcRTx was grown from this starting material). The markers (A) CK8, (B) CK19, (C) CK18, (D) c-met, (E) CD117 and (F) CD133 were used to stain the non-parenchymal cells and the positive controls. For all markers (i) non-parenchymal cell cytospin stained with marker, (ii) non-parenchymal cell cytospin with IgG control, (iii) positive control stained with marker and (iv) positive control with IgG control.

3.5.5.2 NpcRTx colony identification in non-parenchymal cell culture

A colony was identified in one well from the non-parenchymal cell culture. The colony was growing on normal tissue culture plastic with PM. A time course of the well can be seen in Fig. 3-5. On day 7 the cells started to attach. The cells were round, approximately 10-20μ in diameter. On day 12 the cells started to spread out and on day 21 the cells started to proliferate, but the morphology was unclear as they were not confluent. On day 31 the cells were confluent and their cuboidal morphology was clear.

To determine if the cells were proliferating, the area covered by this type of cell was counted daily. The area covered was counted by estimating the number of fields observed down the microscope at x40. The area did not change during the monitoring period of 12 days.

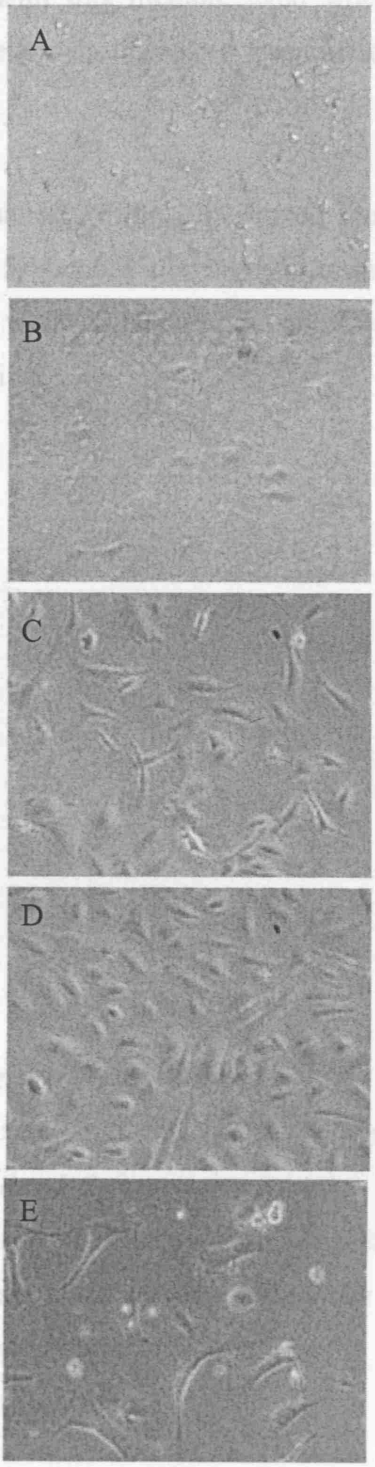


Fig. 3-5 Phase contrast microscopy x200. Time course photographs of the npcRTx colony. (A) day 7, (B) day 12, (C) day 21 and (D) day 31 and (E) day 185.

To determine if more cells were observed within the same area 16 fields inside the colony (x200), in exactly the same area, were photographed every 1 to 3days – a type of slow time lapse photography. The same place in the well was found daily by the use of a scratch at the bottom of the tissue culture plate. When found, the microscope was refocused on the cells and then moved upwards for 16 fields and all fields were

photographed. The colony was observed daily on days 35, 38-42 and 45-46. It was not possible to determine if the cells were proliferating with this method, even when comparing the first and last days of observation. The cells were therefore trypsinised, leaving 1/3 of the cells in the original well.

The observations were started again after trypsinisation. Fig. 3-6 shows four consecutive areas in the well on day 1 (day 48) and day 5 (day 52) after trypsinisation. The pictures are taken from exactly the same place in the well. When day 1 is compared with day 5 it is clear that the cells are proliferating extensively.

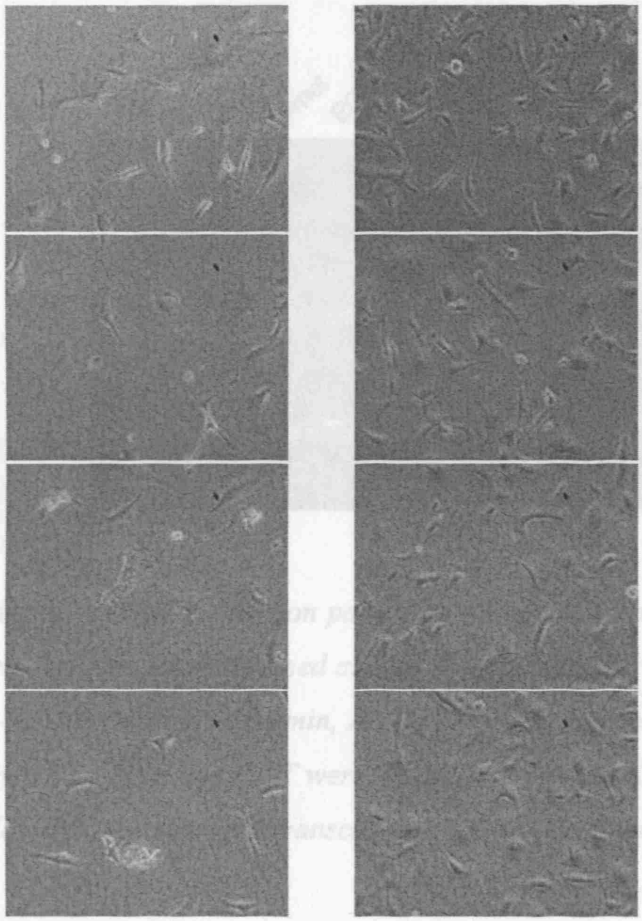


Fig. 3-6 Phase contrast microscopy x200. NpcRTx colony on day 1 (left) and day 5 (right) after trypsinisation. Pictures next to each other represent the same area of the well.

The cells started to senesce on day 185 (Fig. 3-5D). The cells were passaged up to four times before they started to senesce.

3.5.5.3 Gene expression of the npcRTx colony

The cells were analysed for mRNA expression by RT-PCR. The RNA was isolated on day 115. Fig. 3-7 shows the cells expressed the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin and the growth factor receptors c-met, EGF-receptor and TGF-βII. CK18, Cyp1B1 and gamma-glutamyl transpeptidase were also expressed. Albumin, AAT, biliary glycoprotein and alpha-fetoprotein were not expressed. The stem cell transcription factor Oct-4 was observed. However, Oct-4 was also shown to be expressed on HepG2 cells and whole liver [Appendix 2].

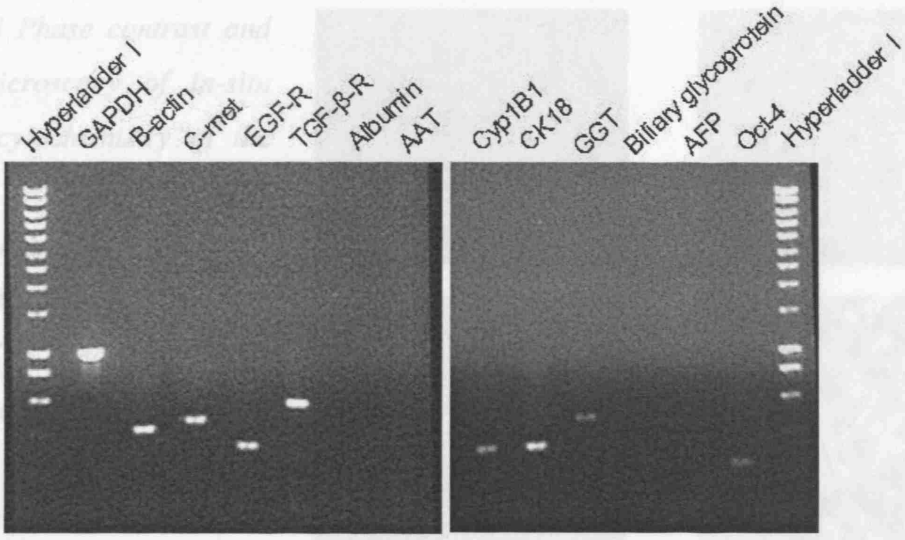


Fig. 3-7 mRNA expression pattern of the npcRTx colony. Housekeeping genes GAPDH and β-actin were expressed as well as growth factor receptors c-met, EGF-R and TGF-β-R. Liver markers albumin, AAT and biliary glycoprotein were not expressed, whereas Cyp1B1, CK18 and GGT were. Fetal liver marker Alpha-fetoprotein was not expressed. However, the stem cell transcription factor Oct-4 was expressed.

3.5.5.4 Markers expressed by the npcRTx colony by immunocytochemistry

In-situ immunocytochemistry were performed on different days to analyse the protein expression by the proliferating population of cells. Fig. 3-8 shows a phase contrast photograph on day 68 and H&E of the same well (day 69). The cells expressed biliary epithelial cell marker CK19 (day 69) and hepatocyte markers CK8 (day 132) and CK18 (day 132). The cells were negative for Human Epithelial Antigen 125 (HEA125) [day 132]. The cells expressed the HGF-receptor c-met (day 123). HGF was therefore added to the culture media of one of the wells, but did not seem to have a proliferative or any

other effect on the cells. However, FBS was already present in the culture media, which might have been a sufficient source of HGF. CD133 was not expressed by the cells, on the other hand CD117 (c-kit, stem cell factor receptor) [day 123] was expressed on some rare cells. All immunocytochemistry assays had negative controls with IgG controls at the same concentration to the antibodies. Positive controls were used alongside to ensure the antibodies worked. Weri-Rb-1 cytospins for CD133, MO7e cytospins or FFPE sections of human appendix for CD117, primary human hepatocytes for CK8 and CK18 and human liver sections for CK19 and HEA125 were used.

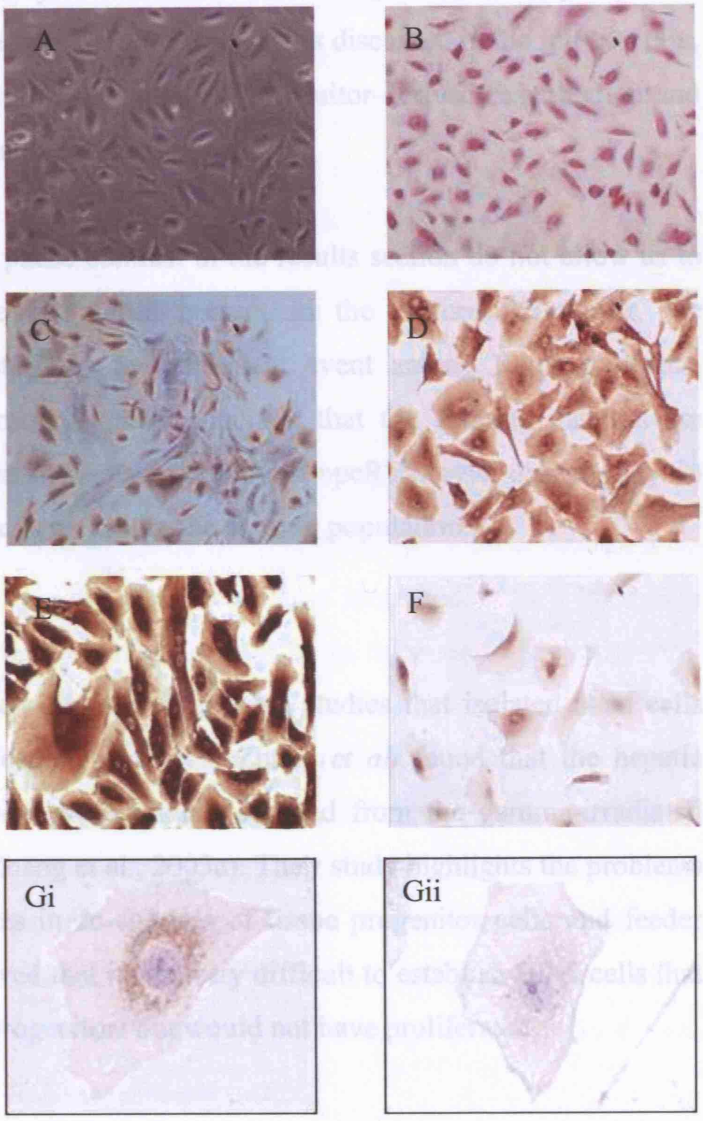


Fig. 3-8 Phase contrast and light microscopy of in-situ immunocytochemistry of the npcRTx colony x200. (A) Phase contrast and (B) H&E. (C) CK19, (D) CK8, (E) CK18 and (F) c-met expression. (Gi) Stem cell marker CD117 and (Gii) negative cell in the same well x400.

3.5.5.5 Protein secretion

ELISA data showed that there was no detectable albumin or alpha-1-antitrypsin in the conditioned media.

3.6 Discussion

The non-parenchymal cell isolation method uses a 50g spin and a 30μm mesh to remove most hepatocytes. The remaining cells include red blood cells, and a mixture of fibroblasts, kupffer cells, stellate cells and endothelial cells. Fibroblasts and stellate cells support the framework of the liver, whereas kupffer cells are liver macrophages. These cell types are very prominent in cirrhotic liver tissue. Endothelial cells line the blood vessels. The lymphoprep gradient was used to select for small cells with a high nucleus/cytoplasm ratio. Moreover, culture of the cells also selected for certain cell population as, for example hepatocytes would have had a very low attachment rate and primary hepatocytes normally survive only for a week. As discussed in the introduction, the diseased livers are thought to be activated for progenitor-derived regeneration and therefore enriched in stem cells and liver progenitors.

The morphologies presented by phase contrast in the results section do not allow us to draw firm conclusions on the cell types present in the cultures. However, the morphologies are useful to determine an infrequent event among frequent events. Moreover, the immunocytochemistry results conclude that the staining patterns are different in typical colonies from the isolated colony in npcRTx, even though they do not provide information on the cells present in the starting population.

3.6.1 Feeder cell layers

Interestingly, it has recently been discovered in some studies that isolated stem cells have been contaminated with feeder layer cells. Zhang *et al.* found that the hepatic progenitor lines from a rat model were actually derived from the gamma-irradiated mouse embryonic feeder cells (Zhang et al., 2003a). Their study highlights the problems associated with possible artefacts in co-cultures of tissue progenitor cells and feeder layer cell lines. Our studies showed that it was very difficult to establish HS-5 cells that would have supported putative progenitors but would not have proliferated.

3.6.2 Why is the colony in npcRTx encouraging?

As a summary, the npcRTx colony expressed c-met, EGF-receptor, TGF-β-receptor II, CK19, CK8 and CK18, gamma-glutamyl transpeptidase and CYP1B1. In addition, it expressed the stem cell markers Oct-4 and CD117. However, it did not express HEA125, biliary glycoprotein, AFP, albumin or AAT.

C-met, the HGF-receptor has been identified as an important marker in liver development and bipotential cells from mouse embryonic liver have been isolated using c-met as a marker (Suzuki et al., 2002). Strong c-met expression of the npcRTx colony was observed on the protein level, compared with the isolated non-parenchymal cells.

Cytokeratin expression in the different cell types of the liver help to define the cells. Adult hepatocytes express CK8 and CK18 only, whereas cholangiocytes express predominantly CK7 and CK19 (Omary et al., 2002). Oval cells are thought to express pre-dominantly CK7 and CK19. Embryonic hepatoblasts, on the other hand, are thought to express CK8, CK18 and CK19. Kupffer cells and endothelial cells express vimentin, whereas stellate cells express vimentin, desmin, nestin and glial fibrillary acidic protein (Omary et al., 2002). The isolated non-parenchymal cells showed no CK19 expression, and only very few CK8 and CK18 expressing cells. This expression was very different from the isolated colony, which had strong CK8 and CK18 expression on almost all cells and CK19 expression on most cells.

CD117 (c-kit), the receptor for stem cell factor (SCF), has been identified on haematopoietic stem cells and putative liver stem cells (Bunting and Hawley, 2003; Crosby et al., 2001). SCF both enhances proliferation and prevents apoptosis in haematopoietic stem cells and is thought to be important for the maintenance of stem cells (Smith et al., 2001). Oct-4, on the other hand, was identified to be a marker of undifferentiated ES cells and has also been identified on haematopoietic stem cells isolated from umbilical cord blood (Reubinoff et al., 2000; Baal et al., 2004). However, it is of intrest that in this study Oct-4 expression was identified by RT-PCR in HepG2 cells and whole human liver. This expression appears to occur in tumour-derived hepatocyte lines, but in whole human liver the source of expression is unclear and it could come from resident progenitor cells. Very few $CD117^{+}$ cells were observed in the npcRTx colony, which is not surprising as CD117 has been found to be lost in culture (Crosby et al., 2001). No CD117 positive cells were detected in the isolated non-parenchymal cells.

The combination of markers expressed by npcRTx colony is thus striking, in the sense that both hepatocyte and biliary epithelial cell markers are expressed. Because of the two sets of markers the cells are not hepatocytes or biliary epithelial cells, but possibly

immature progenitors to the differentiated cell types. This notion is encouraged by the expression of the stem cell markers.

The cells isolated by Selden *et al.* (Selden et al., 2003) secreted albumin and AAT into the conditioned media. The colony of npcRTx, on the other hand, did not, suggesting these cells might be even earlier progenitors as these functional proteins were not produced. This work was presented as a short talk at EASL monothematic conference "Strategies for Liver Support: From Stem Cells to Xenotransplantation" (Laurson et al., 2003).

3.6.3 The importance of GCSF therapy

The explant used to isolate the npcRTx colony was from a patient who had received GCSF therapy. The patient had received GCSF therapy based on the finding that neutrophil function is improved in patients with acute liver failure when they are administered GCSF (Rolando et al., 2000b; Rolando et al., 2000a). GCSF therapy is also used to mobilise haematopoietic stem cells from the bone marrow into peripheral blood (Thomas et al., 2002). It has been reported that in a rat model of fulminant hepatic failure, GCSF stimulated liver regeneration (Theocharis et al., 2003). As liver stem cells may originate from bone marrow, it could be significant that the colony was found in this patient who had received GCSF therapy.

3.6.4 Problems and future direction

There are a number of problems with the approach in this chapter for identifying stem cells. Firstly, the aspect of looking for something atypical is not very feasible in the cell culture system. It is possible that stem cells or progenitors, which do not have a distinct morphology, are ignored. It is clear that a more defined starting population is required.

Secondly, any identified colony is in culture with other cells. This means that any preparation, e.g. cDNA, could contain material from more than one cell colony. Even though the colonies might require other cells for a suitable microenvironment, a means of obtaining a clonal population must be sought.

Thirdly, the cells are relatively slow to grow and they eventually senesce. The identification of a potential progenitor colony requires 3-4 weeks of extensive screening

of a very large number of wells. Once the well is identified, it then requires further passages to obtain enough cells for analysis. At this stage the cells might have been in culture for 2-4 months. The npcRTx colony senesced after 6 months of culture, and during that time the number of cells available for analysis was always limiting. If the colony could be identified earlier, a larger part of the cells life-span could be used more effectively.

These considerations indicated that further work should therefore be directed towards increasing the lifespan of any potential colonies and selecting more defined starting populations.

Chapter 4

In vitro expansion and differentiation studies on non-parenchymal cell colonies

4.1 Introduction

4.1.1 Background

If liver progenitor cells are to be used in a bio-artificial liver device, they need to fulfil certain conditions. Firstly, the cells need to be able to expand to large enough numbers needed for the device. Secondly, these cells need to be able to attain mature differentiated liver cell functions. *Chapter 3 Non-parenchymal cells in culture* reports the identification of a possible progenitor cell colony. However, this population had a limited expansion capacity and therefore differentiation studies were not conducted. This chapter will investigate the *in vitro* expansion and differentiation of identified putative liver progenitor cell colonies.

The introduction is divided into two sections. 4.1.2 considers the possibilities of prolonging the expansion of the colonies, whereas 4.1.3 discusses factors influencing hepatocyte differentiation.

4.1.2 Prolonging the culture of non-parenchymal cells

Immortalisation allows cells to escape the normal limitations on growth and finite number of cell divisions. Immortalisation can occur spontaneously, be induced by mutagens or can be achieved by genetically engineering the cells. Most immortalising methods cause the cells to switch off specific cell functions and only concentrate on growth, i.e. the cells become transformed (Alberts et al., 1994). Due to the requirements of a fully functional liver cell for the bio-artificial liver device, we sought a method for prolonging the culture that would keep the cell cycle and functions as normal as possible. The prevention of cell ageing, by telomere maintenance, has been observed in some stem cells (discussed in section 4.1.2.2) and could be considered to be a relatively natural way of immortalising cells as it is a naturally occurring phenomenon.

4.1.2.1 Cell ageing, telomeres and telomerase

Normal somatic cells have a finite life-span due to cell ageing, caused by telomere shortening. A review by Saldanha *et al.* summarises the assessment of telomere length and factors that contribute to its stability (Saldanha et al., 2003b). **Telomeres are short strands of tandem hexameric repeats (TTAGGG) which cap the ends of linear chromosomes to protect the chromosomes from degradation and prevent end-to-end fusions.** In normal somatic cells, cell division results in the erosion of telomeric repeats and reduction in telomere length due to the 'end-replication problem' (Fig. 4-1). This problem results from the DNA polymerase synthesis being in the 5' to 3' direction and the requirement of an RNA primer to initiate the synthesis. On the lagging strand, a gap is formed where this primer is removed and there is no 3' end to which the next primer can attach. This causes gradual loss of the chromosomal termini at each cell division (Olovnikov, 1973; Dhaene et al., 2000). Short telomeres correlate with reduced cell proliferation and the start of the ageing process (Saldanha et al., 2003b). Telomere attrition in normal somatic cells might be a method of protecting the cell from uncontrolled proliferation (Wong and Collins, 2003).

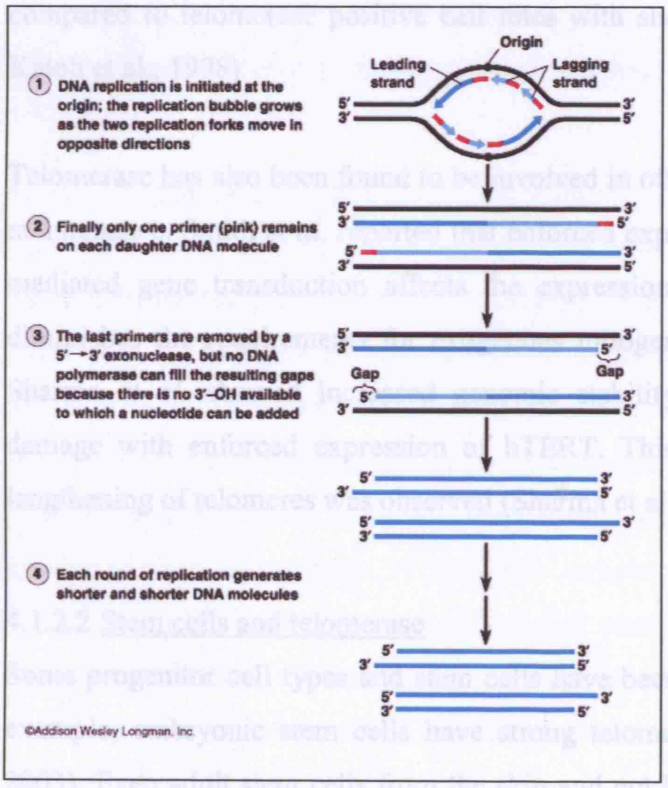


Fig. 4-1 Diagram representing the 'end-replication problem' during DNA replication (MUN, 2005). The gradual loss of telomere length at each cell division is associated with cell ageing.

It was suggested that rapidly proliferating cells, e.g. germline cells, tumour cells and stem cells, possess a particular mechanism for maintaining telomere length. This

mechanism was found to be associated with the activity of the enzyme telomerase (Saldanha et al., 2003b; Saldanha et al., 2003a; Dhaene et al., 2000). **Telomerase can synthesise new telomeres and therefore enables cells to escape the normal attrition of telomeres associated with cell ageing** (Dhaene et al., 2000). Little or no telomerase is expressed in normal somatic dividing cells or pre-senescent cells but telomerase is strongly expressed in tumour and immortalised cells. However, the telomere length in tumour cells is generally shorter than in normal telomerase-positive cells indicating that the telomeres are maintained rather than extended (Saldanha et al., 2003b; Counter et al., 1998a).

Although most immortal cells express telomerase, there are some cells that use other methods for maintaining the length of their telomeres (Reddel et al., 1997; Katoh et al., 1998). These methods are termed 'alternative lengthening of telomeres' (ALT). The mechanisms used for ALT are still unknown. Approximately one quarter of all in-vitro immortalised cell lines are telomerase negative (fibroblast cell lines have ALT more commonly than epithelial cell lines), and even some tumour cell lines were found to be negative. The cell lines not expressing telomerase often have long telomere length compared to telomerase positive cell lines with short telomeres (Reddel et al., 1997; Katoh et al., 1998).

Telomerase has also been found to be involved in other cellular processes than telomere maintenance. Smith *et al.* reported that enforced expression of telomerase by retroviral-mediated gene transduction affects the expression of growth promoting genes and diminishes the requirements for exogenous mitogens (Smith et al., 2003). Moreover, Sharma *et al.* showed increased genomic stability and enhanced repair of genetic damage with enforced expression of hTERT. This was seen before any significant lengthening of telomeres was observed (Sharma et al., 2003).

4.1.2.2 Stem cells and telomerase

Some progenitor cell types and stem cells have been found to express telomerase. For example, embryonic stem cells have strong telomerase expression (Carpenter et al., 2003). Even adult stem cells from the skin and gut have been shown to express low to moderate levels of telomerase (Harle-Bachor and Boukamp, 1996; Kolquist et al., 1998).

Telomerase is also found in haematopoietic stem cells. However, the activity level differs in different subsets and changes with cytokine stimulation (Chiu et al., 1996; Engelhardt et al., 1997). For example, GCSF was found to increase telomerase activity of $CD34^+$ cells derived from bone marrow seven-fold and from peripheral blood fourteen-fold in the same study (Szyper-Kravitz et al., 2003). It has also been noted that telomerase expression in some early progenitors does not prevent telomere shortening completely, but reduces it (Engelhardt et al., 1997; Yui et al., 1998).

However, not all stem cells express telomerase. Human mesenchymal stem cells, for instance, have not been found to express the enzyme (Zimmermann et al., 2003; Banfi et al., 2002). Telomerase might not always be necessary. Telomere attrition only happens when the cell is dividing, but a stem cell, in practice (in spite of its huge potential for cell division) only undergoes a limited number of divisions, and therefore telomere attrition might be minimal (Harrington, 2004). The situation, however, might be different for progenitors. Stem cells divide asymmetrically to produce one stem cell and one differentiating daughter cell. The differentiating daughter cell often produces rapidly proliferating progenitors. Due to their high proliferation rate, they may require telomerase activity and therefore even though the stem cell does not have telomerase activity, the daughter cells might become telomerase positive (Harrington, 2004).

4.1.2.3 Determining telomere length and telomerase activity

Telomeres can be investigated in two ways: by measuring the length of the telomere and by measuring telomerase activity (Saldanha et al., 2003b; Saldanha et al., 2003a; Dhaene et al., 2000). Measuring telomere length involves a comparison of two time points in order to observe telomere attrition or maintenance. It is important to remember that telomerase activity does not correlate with longer telomeres. In fact, immortalised cells often have shorter telomere lengths than normal somatic cells, whilst having greater telomerase activity. Telomerase activity measurements are more easily interpreted as, compared with most normal somatic cells, only immortalised cells express the enzyme. The method of choice for observing telomerase activity is the highly sensitive telomeric repeat amplification protocol (TRAP assay) (Kim et al., 1994; Saldanha et al., 2003a).

4.1.2.4 Restore telomerase activity and the cells escape old age

Telomerase is composed of two main sub-units, the catalytic sub-unit hTERT and the integral template of hTERT, human telomerase RNA (hTR) (Saldanha et al., 2003a). Telomerase inactivation in human somatic cells is mainly due to transcriptional repression of hTERT and alternative splicing (Ulaner et al., 1998). There can be re-activation of telomerase in malignant cancers when there is a loss of tumour suppressor activity or mutations in key tumour suppressor genes (Saldanha et al., 2003a; Dhaene et al., 2000). On the other hand, the transcriptional repression can be overcome by constitutive expression of hTERT, which can be used to immortalise cells (Counter et al., 1998a; Counter et al., 1998b; Bodnar et al., 1998; Wong and Collins, 2003).

Immortalisation, or extended proliferation, using hTERT transduction has been used extensively. Both human bone marrow stromal cells (Simonsen et al., 2002) and $CD34^+$ human cord blood cells have been transduced with hTERT (Elwood et al., 2004). Bone marrow stromal cells achieved extended proliferative life-span and maintained differentiation for osteoblasts. *In vivo* the cells formed bone but did not form tumours. On the other hand, $CD34^+$ cells from cord blood had enhanced long-term survival but no increase in their proliferative capacity.

Matsumura *et al.* established an immortalised, and reversible, human liver endothelial cell line by transducing the cells with both SV40T and hTERT (Matsumura et al., 2004). Furthermore, Wege *et al.* found that hTERT transduction both induced indefinite *in vitro* expansion of foetal human hepatocytes and maintained their differentiation potential (Wege et al., 2003a; Wege et al., 2003b).

4.1.2.5 hTERT insertion using retroviral transduction

Our collaboration with Dr Mark Clements at the Institute of Biomedical Research, UCL, has enabled us to transduce colonies of non-parenchymal cells with the catalytic sub-unit of telomerase, hTERT, using the plasmid construct kindly provided by Dr Robert Weinberg at the Whitehead Institute of Biomedical Reseacrch, MIT. The insertion was carried out using retroviral transduction.

A retrovirus consists of a single stranded RNA genome. The RNA is converted in the cytoplasm into linear double stranded DNA, whereafter it migrates to the nucleus,

circularizes and integrates into cellular DNA. Cells contain 1 to 20 copies of integrated proviral DNA and no specific sites for integration exist, i.e. integration is by non-homologous recombination (Dimmock and Primrose, 1994b; Dimmock and Primrose, 1994a). For a retrovirus to integrate and express viral genes the target cells must be dividing, which limits the number of cells transduced (Cann, 1998).

Some retroviruses contain protooncogenes which can cause cancers, although these are removed in the production of vectors. However, cells can also be transformed due to insertional mutagenesis, where the DNA is integrated near a cellular protooncogene or by disrupting a tumour suppresser gene (Cann, 1998). These considerations are minimal for *in vitro* experiments that do not involve gene therapy for patients.

4.1.3 Differentiation into functional liver cells

Liver specific function is the second important condition for a cell in a bio-artificial liver device. Stem cells and liver progenitors are undifferentiated and will mature to functional hepatocytes with the right cues. *In vivo* differentiation is influenced by a vast array of signals both from the body and from the microenvironment. Extracellular signals, such as cytokines, are also important regulators of cells *in vitro*. It is important to examine foetal liver cell [4.1.3.1] and stem cell [4.1.3.2] culture, as well as liver regeneration studies [4.1.3.3] for a better understanding of differentiation.

4.1.3.1 Foetal liver cell differentiation

Liver development consists of several stages regulated by intrinsic mechanisms and extracellular signals (Kinoshita and Miyajima, 2002). Fibroblast growth factors (FGF) and their receptors have been identified as important in the initial stages of development. Furthermore, transforming growth factor beta (TGFβ) has been found to be important and act synergistically with FGF (Kinoshita and Miyajima, 2002). After the initial stages, hematopoietic cells produce Oncostatin M (OSM) which induces hepatocyte maturation (Kinoshita et al., 1999; Suzuki et al., 2003). At postnatal stages, HGF is produced by non-parenchymal liver cells and is also involved in hepatic maturation (Kamiya et al., 2001; Suzuki et al., 2003). Schmidt *et al.* reported that mice lacking HGF fail to complete development and die *in utero* with reduced size livers showing extensive parenchymal cell loss (Schmidt et al., 1995). Liver cell development

is thus influenced by a number of important growth factors. However, studies have shown that HGF and OSM are central for induction of differentiation *in vitro*.

OSM is an IL-6-type cytokine that causes diverse biological responses in a wide variety of cells. It has potential roles in regulating gene activation, cell survival, proliferation and differentiation (Gomez-Lechon, 1999). OSM binds to β-receptor/gp130 complexes which are expressed in a wide variety of cell types. Signal transduction is mediated by the JAK/STAT tyrosine kinase pathway (Gomez-Lechon, 1999).

HGF is a widely acting glycoprotein, also known as scatter factor, involved in motogenesis, mitogenesis and morphogenesis. HGF binds to its receptor c-met and stimulates the tyrosine kinase activity of the receptor which phosphorylates and activates a number of signal transducers (Stuart et al., 2000). Proliferation of several types of cells *in vitro* has been observed in response to HGF stimulation. Most importantly, HGF is involved (together with other growth factors such as TGFα and EGF) in activating quiescent hepatocytes to proliferate during liver injury (Stuart et al., 2000). Studies in foetal liver cell maturation, using both OSM and HGF, have been carried out both in rodent and human cells and are described below.

Murine foetal liver cells that were cultured for 10 days with OSM in the presence of glycocorticoid showed morphological changes (multiple clusters of cells that resembled hepatocytes were observed) and up-regulation of multiple liver-specific functions (ammonia clearance, lipid synthesis, glycogen synthesis and detoxification) (Kamiya et al., 1999). Moreover, foetal mouse liver cells cultured in the presence of OSM, nicotinamide and DMSO acquired a differentiated morphology (binucleated cells and tight gap junctions between cells) and increased liver functions, such as albumin production and P450IA1/2 activity (Sakai et al., 2002). Terminal differentiation has been found to be influenced by glucocorticoid, high density culture and an extracellular matrix (ECM) (Kinoshita and Miyajima, 2002).

The effects of OSM have also been studied on human foetal hepatocytes. The cells were cultured in the presence of OSM for 30 days (Lazaro et al., 2003). OSM had an effect on both the morphology and protein expression of the cells. Large cells emerged with small vacuoles in the cytoplasm, rich in mitochondria and endoplasmic reticulum. AFP, CK19 and π-GST (foetal isoform) expression were reduced, whereas HNF4α, HepPar1

and α-GST (adult isoform) expression were increased. Furthermore, glycogen deposition and G6Pase (glucose-6-phosphatase) activity increased (Lazaro et al., 2003).

The effects of OSM and HGF together have been studied on foetal mouse liver cells (Kamiya et al., 2001). Kamiya *et al.* cultured the cells in the presence of both OSM and HGF for 7 days. HGF in the presence of dexamethasone induced G6Pase, tyrosine amino transferase and carbamoyl-phosphate synthase and accumulation of glycogen, although to a lesser extent than OSM. Both HGF and OSM upregulated the intracellular production of albumin, but albumin secretion was only observed in response to OSM (Kamiya et al., 2001).

4.1.3.2 Stem cell differentiation into liver cells

Stem cell differentiation into liver cells in response to growth factors has been observed by multiple groups in both rodent and human cells. The origin of the cells, their stem cell markers, the differentiation conditions and liver cell analysis varies to a large degree, making comparisons difficult. Below is a description of the differentiation observed in stem cells derived from foetal mouse liver, rat bone marrow, mouse ES cells and human (rat and mouse) mesenchymal and haematopoietic stem cells.

Suzuki *et al.* showed that hepatic stem cells isolated from foetal mouse livers (c-met^{+}CD49f$^{low/+}$CD117^{neg}CD45^{neg}TER119^{neg}) were induced to albumin expressing, trytophan-2, 3-dioxygenase (TO) positive and G6Pase expressing mature hepatocytes with HGF and OSM in 10 days of culture (Suzuki et al., 2003). HGF (but not acidic FGF and basic FGF) differentiated albumin negative cells into albumin positive cells that also expressed AAT, but not G6Pase and TO. However, OSM induced G6Pase and TO expression and thus promoted maturation of differentiating hepatocytes. AFP and epithelial cell marker (CK19 and GGT) expression were decreased in the presence of either HGF or OSM (Suzuki et al., 2003). Minguet *et al.*, on the other hand, used HGF and OSM to differentiate CD117^{low}CD45^{neg}TER119^{neg} cells isolated from mouse foetal liver. It was found that HGF alone stimulated TTR, albumin and AAT upregulation. OSM alone provided a weaker stimulus, but selectively activated G6P and TAT expression. The two cytokines were found to co-operate in hepatocyte differentiation (Minguet et al., 2003).

Wang *et al.* also showed that rat bone marrow stromal cells cultured with HGF for 10 days were able to produce cells expressing both albumin and AFP (Wang et al., 2004). Hu *et al.* used HGF and aFGF to promote liver differentiation in mouse ES cells. AFP, albumin, CK8 and CK18 were expressed after 11 days in culture (Hu et al., 2003). Oh *et al.* used HGF to differentiate rat bone marrow cells into albumin expressing, $CK8^+$ and $CK18^+$ hepatocyte-like cells in 21 days (Oh et al., 2000).

Schwartz *et al.* induced human, mouse and rat multipotent adult progenitor stem cells (MAPC) to differentiate into hepatocytes *in vitro* (Schwartz et al., 2002). The cells were grown on matrigel with FGF-4 and HGF. The cells started to express HNF-3β, GATA4, CK19, transthyretin and AFP by day 7 and CK18, HNF-4 and HNF-1α on days 14-28. The cells stained positive for albumin and CK18 on day 21. Furthermore, the cells had functional characteristics of hepatocytes, e.g. they secreted urea and albumin, had inducible cytochrome p450 activity, were capable of LDL uptake and stored glycogen. OSM did not increase the percentage of cells positive for these hepatocyte markers. Fiegel *et al.* isolated $CD34^+$ bone marrow cells and cultured them on collagen in the presence of HGF. The cells differentiated to express CK19 and albumin after 28 days of culture (Fiegel et al., 2003a).

4.1.3.3 Liver regeneration studies

During liver regeneration, hepatocytes undergo one or two rounds of replication. EGF, TGFα and HGF have been shown to stimulate DNA synthesis in hepatocytes *in vivo* and *in vitro*. Cultured cells seem to be much more sensitive than quiescent hepatocytes in intact livers to these factors (Fausto et al., 1995). Furthermore, biliary cells have also been found to proliferate *in vitro* when stimulated with HGF (Joplin et al., 1992).

Nakamura *et al.* have investigated the effect of OSM-receptor knockout in mice (Nakamura et al., 2004). The mice had impaired liver regeneration with persistant parenchymal necrosis after CCl_4 exposure. Recovery from partial hepatectomy was also delayed. It was suggested that OSM signalling is required for hepatocyte proliferation and tissue remodelling during liver regeneration (Nakamura et al., 2004).

Shiota *et al.* showed accelerated proliferation of oval cells by transferring HGF into the liver of a 2-AAF/PH rat model (Shiota et al., 2000).

Tomiya *et al.* investigated HGF levels in serum of hepatectomized and non-hepatectomized surgical patients. It was found that the levels were increased in both groups, but hepatectomised patients required 28 days to return to preoperative levels, compared to 7 days for non-hepatectomised patients (Tomiya et al., 1992).

4.1.4 Conclusion

In order to fully explore the potential and differentiation ability of a putative liver stem cell colony, it needs to have an extensive proliferation capacity. Immortalization or prolonging the life-span may be achieved by hTERT transduction. This method has advantages, as an active telomerase is able to maintain the telomere length of cells. This process prevents or delays natural cell ageing of somatic cells but should not interfere with the normal functions of the cells, e.g. differentiation capacity. Differentiation *in vitro* of primary cell cultures is influenced by growth factors and cytokines. Studies from liver development, liver regeneration and *in vitro* studies demonstrate the importance of HGF and OSM as differentiating factors.

4.2 Hypothesis

The hypotheses of this chapter were:

1) The putative progenitor cell population (isolated in *Chapter 3*) does not express telomerase and this contributes to its finite lifespan.
2) Non-parenchymal cell cultures can be transduced with hTERT.
3) Transduced hTERT cells will maintain progenitor cell characteristics and can be induced to differentiate.

4.3 Aims

The aims of this chapter were to:

1) Assess the telomerase expression of the npcRTx colony.
2) Use hTERT transduction to insert the catalytic subunit of telomerase into non-parenchymal cells.
3) Characterise hTERT transduced cell colonies.
4) Investigate the differentiation capability of hTERT transduced cell colonies.

4.4 Methods

4.4.1 Starting material – Non-parenchymal liver samples

Non-parenchymal cells were isolated and cultured from human explant livers as described in *Chapter 2 General Methods*. PM media with no additional growth factors was used. Table 4-1 gives details of the liver samples used.

Table 4-1 Non-parenchymal cell samples used in this chapter.

Disease aetiology	GCSF	Gender	Age
re-transplant	Yes	Female	51yrs
ALD	No	Male	46yrs
cryptogenic	No	Male	53yrs
fulminant	No	Female	35yrs

4.4.2 Positive controls

Positive controls were used for immunocyto/histochemistry and RT-PCR analysis.

4.4.2.1 Human liver tissue

Human liver tissue was used as a positive control for all liver markers for RT-PCR and immunohistochemistry. For more detailed methods please refer to *Chapter 2 General Methods*.

4.4.2.2 Weri-Rb-1

Weri-Rb-1 cells express CD133 and were used as a control for experiments assessing this antigen.

4.4.2.3 MO7e

MO7e cells express CD117 and were used as a control for experiments observing this antigen.

4.4.2.4 HepG2

HepG2 cells express c-met and were used as a control for experiments observing this antigen.

4.4.3 Negative controls

Non-specific IgG controls at the specific antibody concentrations were used to confirm low non-specific binding of the antibodies used for immunocyto/histochemistry.

4.4.4 Telomerase activity –TRAP assay

Telomeric repeat amplification protocol (TRAP assay) with TeloTAGGG Telomerase PCR ELISA Plus kit (Roche cat no2013789) was used, kindly provided by Dr Mark Clements. The TRAP assay is a photometric enzyme immunoassay for quantitative determination of telomerase activity. The assay relies on telomerase-reaction products that are produced in the presence of telomeric repeats and primers if the samples contain telomerase. The telomerase-reaction product was amplified by PCR. For ELISA detection, the PCR products were split into two aliquots, denatured and hybridised separately to digoxigenin-(DIG)-labelled detection probes, specific for the telomeric repeats and for the internal standard. The resulting products were immobilised via the biotin label to a streptavidin coated microtiter plate. Immobilised amplicons were then detected with an antibody against DIG conjugated to horseradish peroxidase (a-DIG-HRP) and the sensitive substrate TMB (containing 3,3',5,5'-tetramethylbenzidine). The method is ideal for a small number of cells. All samples had two internal controls. Heat inactivated sample (negative control) and internal standard (positive control) for a different target than telomerase to control for the amplification step. For more detailed methods, please refer to *Chapter 2 General methods*.

4.4.5 Transduction with hTERT

This part of the project was performed at the Wolfson Institute of Biomedical Research under the supervision of Dr Mark Clements. All plasmids and reagents were prepared in their laboratory. The vector construct pBabe-hEST2 was kindly provided by Dr Robert Weinberg at the Whitehead Institute of Biomedical Research, MIT (Counter et al., 1998b; Counter et al., 1998a). The vector maps for hTERT and GFP are shown in Appendix 3.

4.4.5.1 Producing virus particles

For safety purposes the virus particles are not able to replicate. Therefore, to produce virus particles 293gp was used as a packaging cell line. 293gp is a human kidney cell

line expressing viral gag (coding for core proteins) and pol (coding for reverse transcriptase) that are required for retroviral replication [Clontech BD].

Vector plasmid (pBabepuro-EST2 for hTERT or pHMIV-eGFP for GFP control) was co-transfected into 293gp cells with packaging plasmid VSVG (enabling transduction of a broad range of host cells) using a calcium phosphate method. Virus was collected after three days. For details see *Chapter 2 General methods.*

To assess transfection efficiency of the method, GFP 293gp cells were observed under a fluorescent microscope and analysed by flow cytometry when the viral supernatant was collected.

4.4.5.2 Transducing non-parenchymal cells

The non-parenchymal cells were incubated for 8hrs with the viral supernatant. After six days, the transduction was repeated with the frozen virus particles. For more detailed methods, please refer to *Chapter 2 General methods.*

The hTERT expressing cells were selected with puromycin on two different occasions (day 63 and 151) after transduction. The GFP cells were used as a control for puromycin selection efficiency, as the plasmid with the GFP did not contain a puromycin resistance gene. All cells transduced with GFP died and lifted off within 5 days of selection.

4.4.6 Culture on fibronectin-coated tissue culture plates

Cells were plated onto fibronectin-coated tissue culture plates and cultured for 28 days. When cells needed to be sub-cultured they were replated onto new fibronectin-coated plates. Fibronectin-coating is described in *Chapter 2 General methods.*

4.4.7 Gene expression by Reverse Transcriptase PCR

Methods as described in *Chapter 2 General methods.*

4.4.8 Protein expression patterns by immunocytochemistry

Cytospin preparation and staining methods as described in *Chapter 2 General methods*. Anti- CK8, CK18, CK7, CK19 and c-met antibodies were used for all samples.

4.4.9 Enzyme-Linked ImmunoSorbent Assay (ELISA)

Alpha-1-antitrypsin and albumin ELISA as described in *Chapter 2 General methods*.

4.4.10 Differentiation studies with HGF and Oncostatin M

For hepatocyte differentiation, HGF and Oncostatin M (R&D Systems cat no 295-OM) were added to the culture media. The cells had been cultured for 159 days after hTERT transduction before the differentiation studies.

Experimental plan:

Transduced cells were seeded by passaging the cells 1:2 and were allowed to attach with no interference. The following day the media was replaced with one of the four conditions.

1) Control with PM
2) PM + 20ng/ml HGF
3) PM + 10ng/ml Oncostatin M
4) PM + 20ng/ml HGF + 10ng/ml Oncostatin M

Media was changed and collected every two days. The cells were trypsinised as described previously (*Chapter 2 General methods*) if they reached confluency. The analysis included observations every two days, focusing on differences in the rate of proliferation and morphology. The cells were harvested at the end of the experiment for mRNA expression analysis by RT-PCR.

4.5 Results

4.5.1 Telomerase activity in npcRTx

All the cells isolated from npcRTx [*Chapter 3*] had senesced after approximately 6 months of culture. There are two possible explanations. Either the cells were not immortal and therefore, after a certain number of cell divisions, the cells died due to cell ageing; or the other cell types present in the wells might be providing the colony with an essential microenvironment. This microenvironment would consist of, for example, cell-to-cell contact and growth factors. These supporting cells might be lost due to cell ageing and therefore indirectly affect the survival of the colony even if it were immortal.

To investigate if the cells in the npcRTx colony were senescing because of cell ageing, the TRAP assay was used to determine telomerase expression. Telomerase expression is an indication of immortality as explained in the introduction [4.1.2].

The absorbance at 450nm of each sample was compared to the heat inactivated negative controls and were either found to have active telomerase (+) or not to have active telomerase (-).

The HepG2 sample was positive for telomerase and NpcRTx sample was negative for telomerase as seen in Table 4-2. The heat inactivated negative controls were negative for both samples. The internal standards, used as controls for the internal amplification, were positive for both samples, ruling out any inhibition of the assay, which could give false negatives. The negative control and control template (containing DNA with the same sequence as a telomerase product with 8 telomeric repeats) of the assay worked as expected. The lack of telomerase in the npcRTx colony suggested that telomere shortening might be the reason for senescence.

Table 4-2 Telomerase activity assessed by the TRAP method.

	Telomerase activity	Internal standard	Heat inactivated control
HepG2	+	+	-
npcRTx	-	+	-
Control template	+	n/a	n/a
Negative control	-	n/a	n/a

4.5.2 Transduction of non-parenchymal cell colonies

4.5.2.1 Non-parenchymal cell colonies and viral particles

Cells from three explants (ALD, cryptogenic and fulminant liver failure) were cultured and observed. Five wells were chosen with unusual colonies. These colonies were epithelial-type and non-fibroblastic. Fig. 4-2 shows two colonies identified from the fulminant liver failure explant and three derived from the ALD. No colonies were chosen from the cryptogenic explant.

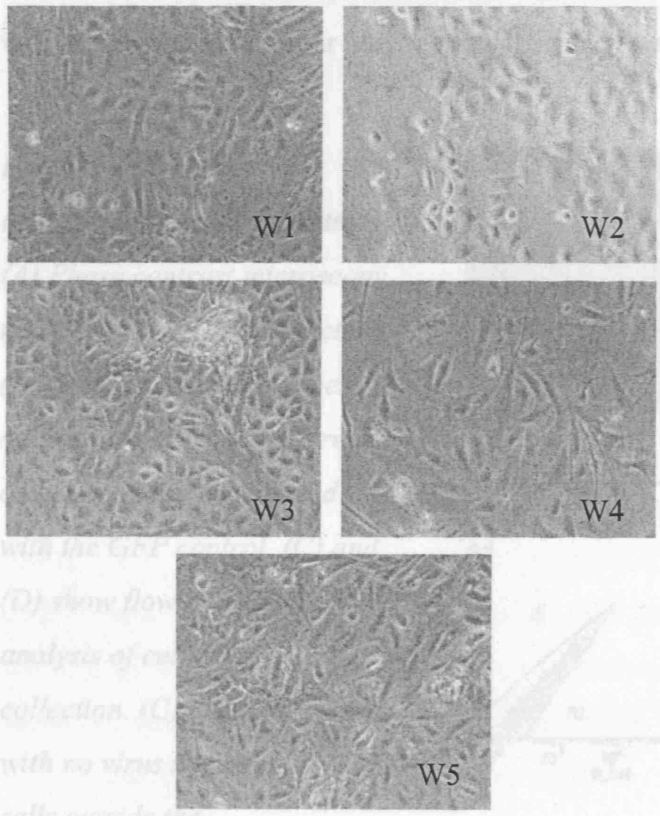


Fig. 4-2 Phase contrast microscopy x200 of colonies chosen (due to their epithelial-type morphology) to be transduced. W1 and W2 isolated from a fulminant liver failure explant and W3, W4 and W5 from an ALD explant.

The five colonies, shown in Fig. 4-2, were transduced by hTERT, the catalytic subunit of telomerase. The fulminant liver failure colonies were transduced on day 71 and ALD colonies on day 79 in culture.

Retroviral particles encoding hTERT were produced by the 293gp packaging cell line after $CaCl_2$ transfection with the plasmids pBabepuro-EST2 for hTERT and pHMIV-eGFP for the GFP control. The viruses were produced simultanously and the GFP construct was used as a control for transfection and transduction efficiency.

To check transfection efficiency of the 293gp cells, the GFP controls were observed under the fluorescence microscope and the percentage of GFP positive cells was quantified by flow cytometric analysis (Fig. 4-3). A high percentage of cells were glowing green under fluorescent microscopy, indicating GFP expression due to successful transfection. The flow cytometry data indicated that 99.72% of the cells were expressing GFP compared with the untransfected control with only 0.03% of the cells being gated as GFP positive. The high transfection efficiency of the 293gp cells suggested that high viral yields would be produced and hence the viral supernatant was used for the transduction of the non-parenchymal cells. The efficiency of the hTERT transfection could not be quantified since the vector did not encode GFP and therefore it was assumed that a similar transfection efficiency was obtained.

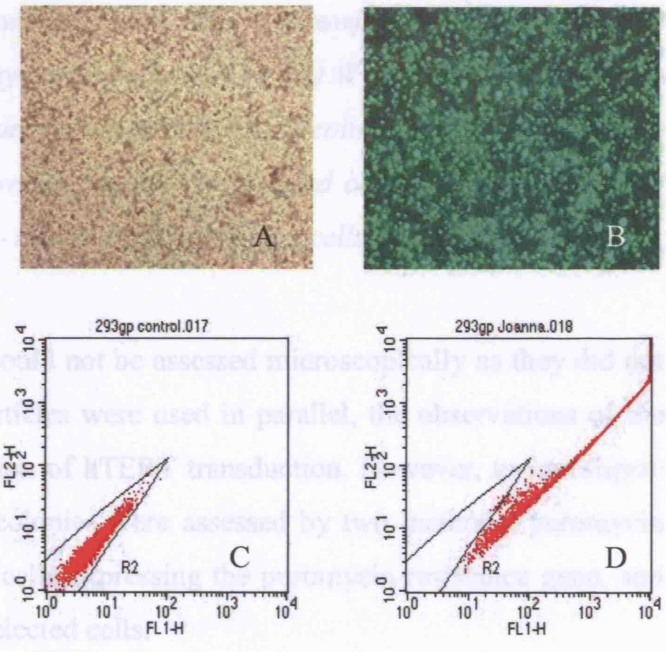


Fig. 4-3 293gp cells transfected with GFP control. (A) Phase contrast microscopy (x200) day 1 after transfection. (B) Area A under fluorescent microscopy (x200). The green cells have been transfected with the GFP control. (C) and (D) show flow cytometric analysis of cells after viral collection. (C) Control sample with no virus showing 0.03% of cells outside the autoflourescence gate. (D) GFP sample showing 99.72% GFP expressing cells.

4.5.2.2 Transduction efficiency of non-parenchymal cells

4.5.2.2.1 GFP vector expression

To assess transduction efficiency, the GFP controls were observed on day 4 after the first transduction (Fig. 4-4). All wells contained green cells indicating transduction. However, more fibroblast-like cells seemed to be tranduced than cuboidal cells. As the retrovirus only transduces dividing cells, the high proliferation rate of the fibroblasts may explain this difference. Moreover, fibroblast may be better at expressing the GFP, causing them to be brighter than the cuboidal cells.

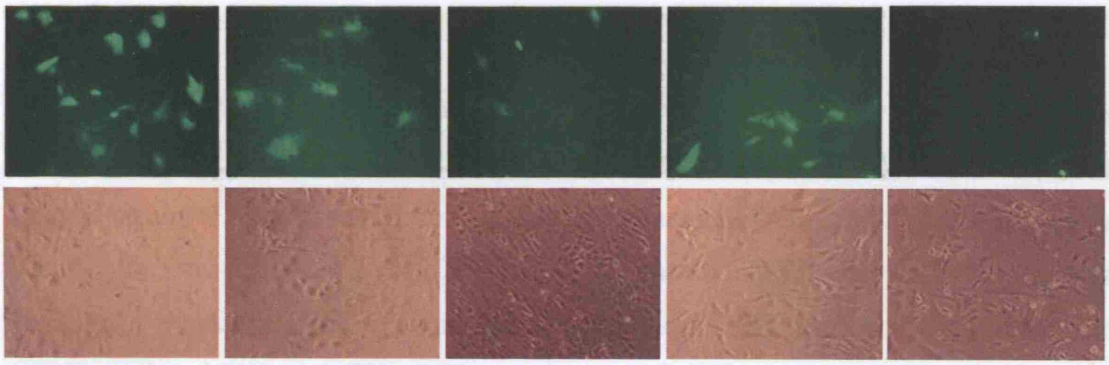


Fig. 4-4 Phase contrast [bottom row] and fluorescent [top row] microscopy x200. GFP control wells for all colonies on day4 after transduction. (A) W1 fulminant liver failure colony, (B) W2 fulminant liver failure colony, (C) W3 ALD colony, (D) W4 ALD colony and (E) W5 ALD colony. All the wells contained transduced cells. The morphology of the cells varied and both fibroblast- like and epithelial-type cells were transduced.

The level of hTERT transduction could not be assessed microscopically as they did not contain GFP. As the two virus particles were used in parallel, the observations of the GFP control wells gave an indication of hTERT transduction. However, to investigate the hTERT colonies directly, the colonies were assessed by two methods; puromycin selection, used to enrich for those cells expressing the puromycin resistance gene, and hTERT mRNA expression of the selected cells.

4.5.2.2.2 Puromycin selection

Puromycin selection of the hTERT transduced cells on day 63 (post transduction), resulted in varying percentages of cells surviving in the wells. Approximately 50% of the cells remained viable in W5, 40% in W4 and 35% in W3. These wells recovered

after the selection pressure was removed. The selection was repeated on day 151 (post transduction). W3, W4 and W5 were all isolates from the ALD explant.

On the other hand, W1 and W2 isolated from the fulminant liver failure explant contained only 20% of viable cells. Moreover, even though these cells could be passaged for a further two passages they finally senesced. Culture was terminated at day 172 post-transfection. These results suggest that a very low number of cells were adequately transduced in W1 and W2. Section *4.5.3 Long term culture* discusses the growth curves of the colonies in more detail.

4.5.2.2.3 hTERT expression

RNA was isolated from hTERT transduced W3, 4 and 5 on day 115 post transduction and after puromycin selection. RNA from GFP transduced W3, W4 and W5 was isolated on day 75 post transduction. cDNA was generated from the RNA and used as a template for primers specific for hTERT. Fig. 4-5 shows hTERT specific PCR products for hTERT transduced W3, W4 and W5. There is no hTERT specific PCR product for GFP transduced W3 and W4. GFP transduced W5, however, shows a faint hTERT specific band. Due to this unexpected band, the PCR was repeated with only the GFP controls. The faint band remained in sample W5. All –RT controls are negative. Fig. 4-5 also shows bright GAPDH specific bands for all samples.

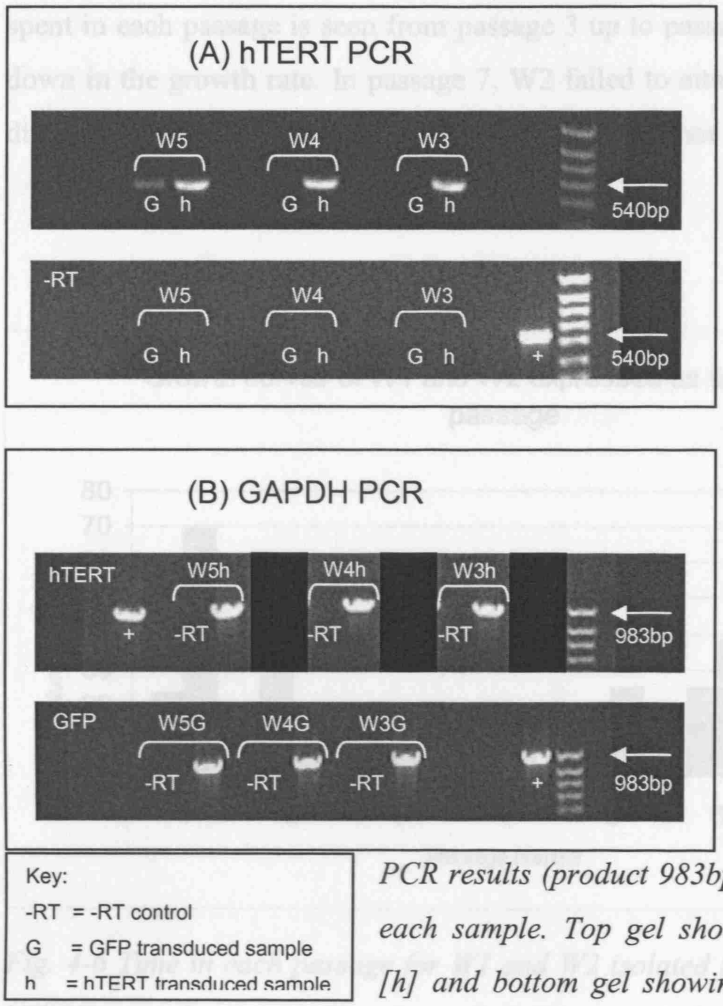


Fig. 4-5 PCR gels showing hTERT and GAPDH expression in W3, W4 and W5.

Panel (A) showing hTERT PCR results (product 540bp). Top gel showing hTERT specific bands for hTERT transduced [h] W3, W4 and W5. No bands were seen in GFP transduced [G] W3 and W4, whereas W5 shows a weak band. Bottom gel shows –RT controls for all samples.

Panel (B) showing GAPDH PCR results (product 983bp). –RT controls on the left of each sample. Top gel showing hTERT transduced cells [h] and bottom gel showing GFP transduced cells [G]. All samples have bright GAPDH bands. Positive control (+) for GAPDH is HepG2 cDNA.

4.5.3 Long term culture

4.5.3.1 Growth rates

Active telomerase acts to restore telomeres that are degraded due to cell ageing. Immortalisation of some cell types have been observed by hTERT transduction and prolonged life-span in others [*4.1.2*]. Out of the five colonies transduced, three colonies (W3, W4 and W5) were found to be puromycin resistant and expressing hTERT mRNA. The long term survival was tracked for over 600days.

Firstly, Fig. 4-6 shows the growth curves of W1 and W2. A gradual increase in the time spent in each passage is seen from passage 3 up to passage 7, demonstrating a slowing down in the growth rate. In passage 7, W2 failed to attach after trypsinisation and was discarded. However, the cells in W1 attached but did not survive.

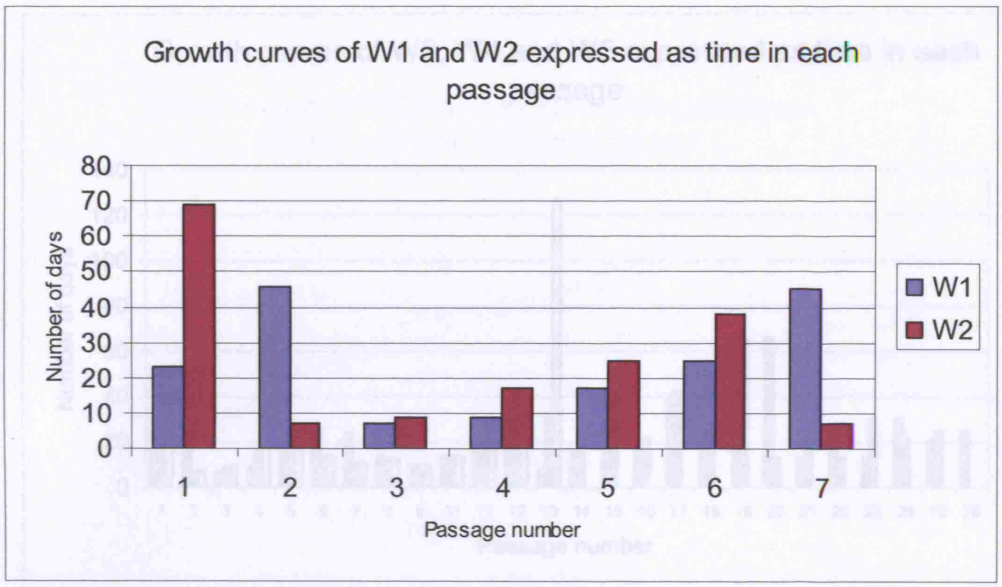


Fig. 4-6 Time in each passage for W1 and W2 isolated from a fulminant liver explant. hTERT transduction was performed at passage 4 for W1 and passage 3 for W2. Puromycin selection occurred during passage 6 for W1 and passage 5 for W2. A gradual lengthening of time spent in each passage is observed from passage 3 to passage 7. A short time in passage 7 with W2 was due to cells failing to attach after trypsinisation.

On the other hand, Fig. 4-7 shows the growth curves of W3, W4 and W5. W5 lasted for 13 passages, where the growth was arrested, but the cells survived in a quiescent state for a prolonged time. A generalised wave-pattern can be observed with the cells, where the cells grow more slowly for a couple of passages followed by quicker growth in the next few passages.

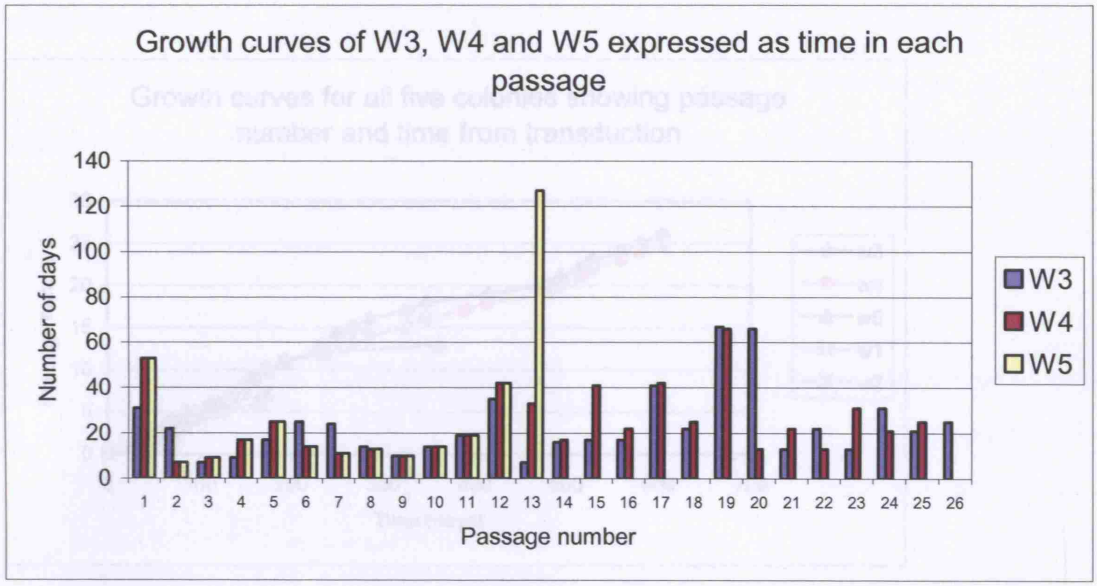


Fig. 4-7 Time in each passage for W3, W4 and W5. hTERT transduction occurred at passage 4 for W3 and passage 3 for W4 and W5. First puromycin selection occurred during passage 6 for W3 and passage 5 for W4 and W5. Second puromycin selection occurred during passage 12 for W3 and passage 11 for W4 and W5. W5 was discarded after 127 days in in passage 13. The cells survived for a long time in culture without growing.

Fig. 4-8 represents the same data plotted as passage number versus time from cell isolation. W1 and W2 senesced 127 days after isolation, whereas W5 survived for 361 days. W3 and W4 were maintained for over 600 days. In passage 12 a decrease in growth rate was observed for W3, W4 and W5 (Fig. 4-7). The time in that passage increased to around 40 days. This trend is seen on Fig. 4-8 just before 200 days where the slope of the graphs decrease.

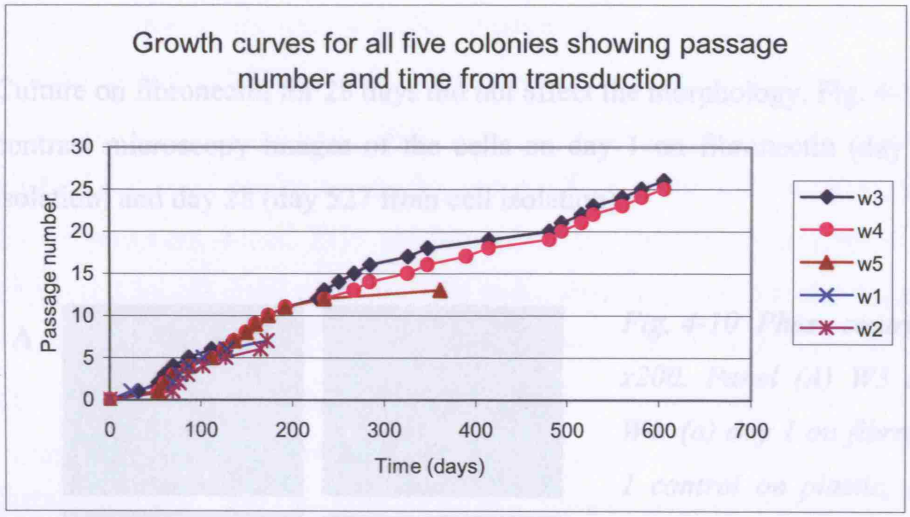


Fig. 4-8 Growth curves for W1-W5 represented as passage number against time from cell isolation. W1 and W2 survived to day 127 and W5 to day 361. A slowed down growth rate was observed from day 192, which can be seen in a decreased slope of the graphs.

4.5.3.2 Morphology

The morphology of the colonies changed progressively during culture. Fig. 4-9 shows the morphology of W3, W4 and W5 on day 206, which can be compared with Fig. 4-2 when the cells were isolated. For W3 and W4 morphology on day 499 and 527 can be seen in the controls in Fig. 4-10 (right hand side). It is clear that the cells have largely lost their cuboidal-type morphology.

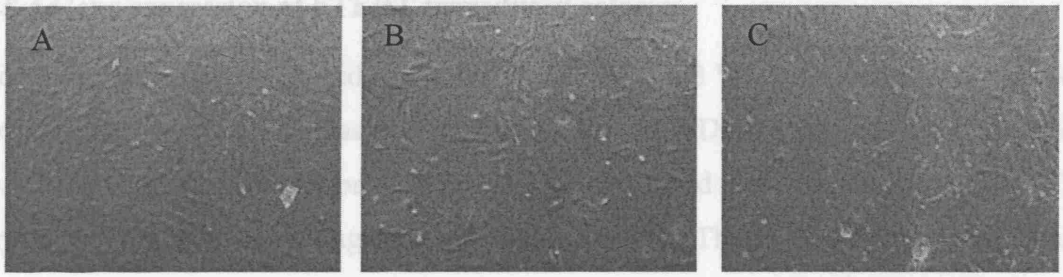


Fig. 4-9 Phase contrast microscopy x200. hTERT transduced colonies on day 206 from cell isolation (A) W3, (B) W4 and (C) W5.

Culture on fibronectin for 28 days did not affect the morphology. Fig. 4-10 shows phase contrast microscopy images of the cells on day 1 on fibronectin (day 499 from cell isolation) and day 28 (day 527 from cell isolation).

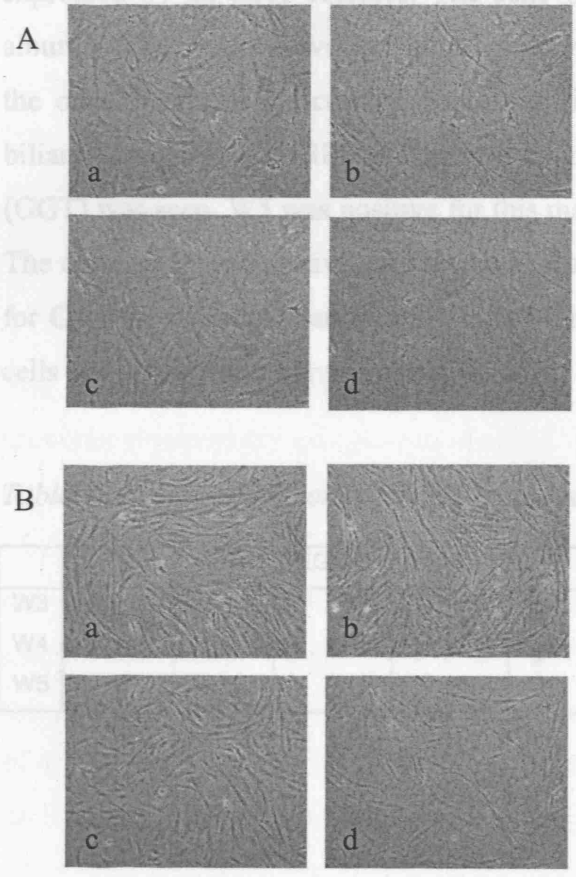


Fig. 4-10 Phase contrast microscopy x200. Panel (A) W3 and panel (B) W4. (a) day 1 on fibronectin, (b) day 1 control on plastic, (c) day 28 on fibronectin and (d) day 28 control on plastic.

4.5.4 Gene expression of hTERT transduced colonies

To characterise the transduced cell colonies, W3, W4 and W5 were analysed for mRNA expression patterns. RNA was isolated on day 115 and cDNA generated from the RNA was used as a template for primers specific for liver and stem cell markers. The PCR products were analysed by agarose gel electropheresis. The PCR gels for each marker are found in Appendix 4. The results are summarised in Table 4-3 and Table 4-4.

Table 4-3 shows that all three colonies express mRNA for the housekeeping gene GAPDH. Due to the cell-to-cDNA method used, the RNA could not be quantified. Instead, bright GAPDH bands were assumed to provide similar amounts of starting material (semi-quantitative) when used at high cycle numbers. The cell-to-cDNA method was used due to continuity and small numbers of cells from the start. Growth factor receptors c-met, EGF-receptor (EGF-R) and TGFβ-II-receptor (TGFβ-R) are expressed by all three colonies. The cells were negative for one hepatocyte marker, albumin (alb), but positive for alpha-1-antitrypsin (AAT) and cytochrome CYP1B1. On the other hand, all the colonies were negative for the biliary cell associated marker biliary glycoprotein (BGP). Differential expression of gamma-glutamyl transpeptidase (GGT) was seen. W5 was positive for this marker, whereas W3 and W4 were negative. The colonies were negative for foetal cell marker alpha-fetoprotein (AFP), but positive for Oct-4, a stem cell transcription factor (although Oct-4 is also expressed in HepG2 cells and whole human liver [Appendix 2]).

Table 4-3 Gene expression of hTERT transduced colonies W3, W4 and W5.

	GAPDH	c-met	EGF-R	TGFβ-R	alb	AAT	CYP1B1	BGP	GGT	AFP	Oct-4
W3	+	+	+	+	-	+	+	-	-	-	+
W4	+	+	+	+	-	+	+	-	-	-	+
W5	+	+	+	+	-	+	+	-	+	-	+

Table 4-4 shows cytokeratin mRNA expression. All wells expressed CK18. W3 and W4 expressed CK7, whereas all wells were CK19 negative.

Table 4-4 Cytokeratin gene expression of hTERT transduced colonies W3, W4 and W5.

	CK7	CK19	CK18
W3	+	-	+
W4	+	-	+
W5	-	-	+

In summary, all colonies exhibited liver markers expressed on both hepatocytes and biliary epithelial cells. Only W5 expressed GGT. All colonies were stem cell transcription factor Oct-4 positive. Cell colonies were negative for albumin, but positive for AAT.

These results suggested that the cell colonies did not have a unique hepatocyte or biliary epithelial type mRNA profile, even though they expressed specific liver markers. Protein expression by immunocytochemistry and ELISA was also investigated [4.5.5 and 4.5.6].

4.5.5 Cytokeratin and c-met expression by immunocytochemistry

The protein expression of W3, W4 and W5 were investigated using immunocytochemistry on cytospin slides of the cells. Cytokeratin markers were used to determine if the cells had a clear liver phenotype. CK7 and CK19 are biliary markers, whereas CK8 and CK18 are hepatocyte markers (Omary et al., 2002). C-met (HGF-receptor) was investigated in order to determine if HGF could be used in differentiation studies. Microscope images of the slides can be seen in Fig. 4-11. Table 4-5 summarises the results. Each colony expressed a different pattern of cytokeratin markers suggestive of different phenotypes. Moreover, some markers were expressed on only some cells in each colony. This might be due to a heterogenous population of cells in the well or some cells differentiating and therefore losing or gaining expression of a cytokeratin. All colonies were c-met positive and CK19 negative. CK7 was expressed by W3 and W4, but not by W5. CK8, on the other hand, was expressed by W4 and some cells in W3 and W5. CK18 was expressed by W3 and W4 and by some cells in W5. These results correspond with the mRNA data.

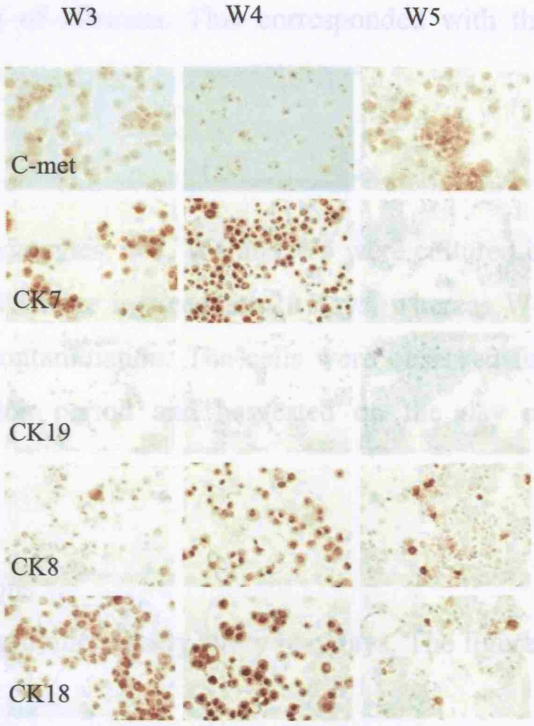


Fig. 4-11 Immunocytochemistry on cytospin slides of W3, W4 and W5. Light microscopy x200. First column W3, second column W4 and last column W5. Markers from top to bottom: c-met, CK7, CK19, CK8 and CK18. All colonies were positive for c-met and negative for CK19. W3 was positive for CK7 and CK18 and had CK8 positive and negative cells. W4 was positive for CK7, CK8 and CK18. W5 was negative for CK7 and had positive and negative cells of both CK8 and CK18.

Table 4-5 Protein expression of hTERT transduced colonies W3, W4 and W5.

	c-met	CK7	CK19	CK8	CK18
W3	+	+	-	+/-	+
W4	+	+	-	+	+
W5	+	-	-	+/-	+/-

4.5.6 Protein secretion by ELISA

In order to assess liver specific protein secretion, alpha-1-antitrypsin (AAT) and albumin (Alb) was analysed from conditioned media by ELISA.

AAT, which was expressed on the mRNA level, was sampled on day 115 and 165 (post transduction) for W3, W4 and W5. None of the samples contained measurable levels AAT. Even though mRNA was expressed, the protein levels were too low to be measured by ELISA.

The conditioned media was assayed for albumin on day 115 for W3, W4 and W5. None of the samples contained detectable levels of albumin. This corresponded with the mRNA results.

4.5.7 Differentiation with HGF and OSM

To induce differentiation into functional hepatocytes, W3, W4 and W5 were cultured in the presence of HGF and OSM. W3 and W5 were induced for 25 days, whereas W4 culture was terminated on day 12 due to contamination. The cells were observed for morphological changes during the induction period and harvested on the day of termination for mRNA analysis.

4.5.7.1 Phase contrast microscopy observations

Changes in morphology were observed by light microscopy every two days. The figures are found in Appendix 5.

4.5.7.1.1 W3

The well containing OSM alone seemed to grow more rapidly, becoming confluent twice (day10 and day22) rather than once as the other wells (day18) during culture. No clear morphological changes could be seen between the different conditions.

4.5.7.1.2 W4

The growth rate of the cells remained similar until they were harvested on day 12 due to fungal contamination. No clear morphological changes were seen between the different conditions.

4.5.7.1.3 W5

Morphological differences were observed after day 6 in culture. On day 6 the cells cultured in OSM had fewer thin, elongated fibroblast-type cells compared with the other conditions [Fig. 4-12, Row 1]. On day 8 a clear difference could be seen between OSM containing wells (OSM alone and OSM+HGF) and the control and HGF treated wells [Fig. 4-12, Row 2]. The OSM containing wells were both more confluent and included cells that were less stringy and had a more cuboidal-type morphology. This effect was more marked in the well containing OSM alone. The morphological differences were

clear until day 16 [Fig. 4-12, Row 3], whereafter the cells became more fibroblastic once again maybe due to the differences being less obvious when the well is confluent. This was more marked in the well containing both OSM and HGF, whereas the well containing OSM alone remained relatively distinct from the other wells [Fig. 4-12, Row 4]. The difference in morphology may be generated by the extensive proliferation of the cells rather than any functional differentiation. No clear differences between the control and the well containing HGF were seen.

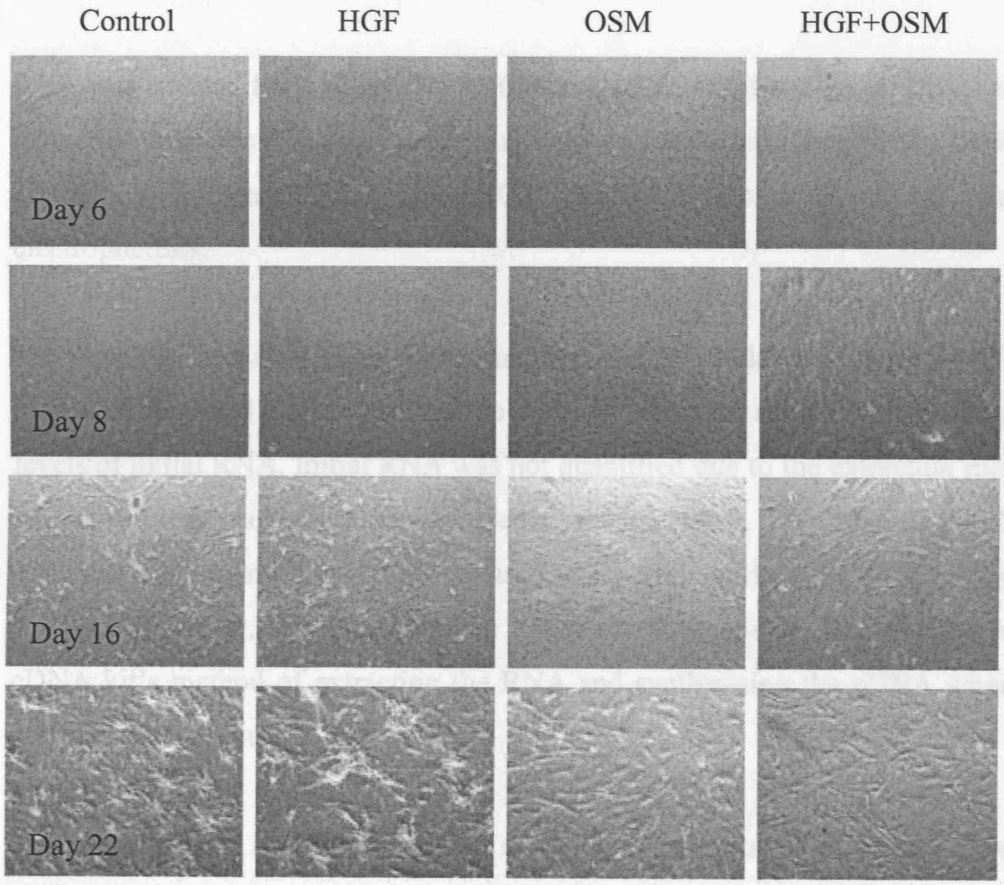


Fig. 4-12 Phase contrast microscopy of differentiation studies of W5 x200. W5 observed on day 6 (row 1), day 8 (row 2), day 16 (row 3) and day 22 (row 4). Control with no additional growth factors in column 1, column 2 with HGF, column 3 with OSM and column 4 with HGF+OSM. Differences in morphology appeared at day 6 in culture. OSM containing wells have fewer fibroblast-type cells and more cuboidal-type cells. Differences were observed until day 16, whereafter the differences were less striking.

4.5.7.2 mRNA analysis of cells induced to differentiate

The mRNA expression patterns from W3, W4 and W5 were examined [Table 4-3 and Table 4-4] in order to identify the most important markers to be studied for the differentiation analysis. Albumin (which was found to be negative in all W3, W4 and W5) was chosen as a good marker for differentiation as HGF and OSM were hoped to induce albumin expression. CK7 and CK19, biliary epithelial cell markers were also chosen, as their expression was expected to change during differentiation. CK7 was expressed by W3 and W4 but not by W5, whereas CK19 was not expressed by any of the colonies.

RNA was isolated from all conditions (control, HGF, OSM and HGF+OSM) and cDNA generated from the RNA was used as a template for primers specific for GAPDH, 18s, albumin, CK7 and CK19. The PCR products were analysed by agarose gel electropheresis.

All samples were GAPDH positive and the –RT controls were negative [Fig. 4-13]. However, the band intensity seemed to differ between the samples, suggesting different levels of initial RNA. Initial RNA was not quantified due to the extraction method used where protein contamination prevents measurement. A Nanodrop (Nanodrop Technologies, cat no ND-1000) was used to try to quantify the cDNA in the different samples. However, this proved to be unsuccessful, probably also due to the cell-to-cDNA kit's method of extracting the RNA and synthesising the cDNA where protein and salts are present.

To investigate the amount of starting material, the amount of 18s was assayed. 18s is preferred to the housekeeping gene GAPDH, which has been shown to vary under different conditions (Suzuki et al., 2000b). Furthermore, 25 cycles were used instead of 40 cycles in order keep the reactions unsaturated. The results are shown in Fig. 4-13 and show very similar patterns of band intensity between GAPDH and 18s. Table 4-6 shows densitometry of the 18s bands. The samples with the low intensity bands are W3 with HGF, W3 with HGF+OSM, W4 with HGF+OSM, W5 control, W5 with OSM and W5 with HGF+OSM. These samples probably have less starting material compared with the other bands and this should be kept in mind for the analysis.

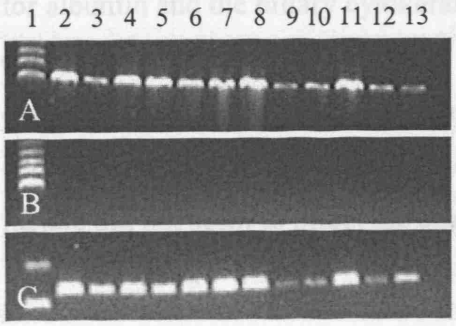


Fig. 4-13 PCR gel showing GAPDH (A), -RT (B) and 18s (C) expression for differentiation experiment of W3, W4 and W5. From left to right – Hyperladder IV (1), W3 control (2), W3&HGF(3), W3&OSM (4), W3&HGF+OSM (5), W4 control (6), W4&HGF (7), W4&OSM (8), W4&HGF+OSM (9), W5 control (10), W5&HGF (11), W5&OSM (12) and W5&HGF+OSM (13). Note the difference in band brightness and smaller bands in lanes 3, 5, 9, 10, 12 and 13. 18s PCR was repeated and showed a similar pattern.

Table 4-6 Densitometry of 18s bands of the samples analysed for the differentiation experiment. 'Lanes' column refers to the gel in Fig. 4-13.

	Optical Density of 18s (25x)	
	PCR bands	Lanes
W3 control	99.5	2
W3&HGF	75.3	3
W3&OSM	86.8	4
W3&HGF+OSM	77.0	5
W4 control	93.2	6
W4&HGF	95.0	7
W4&OSM	88.9	8
W4&HGF+OSM	57.1	9
W5 control	56.3	10
W5&HGF	80.9	11
W5&OSM	51.7	12
W5&HGF+OSM	52.8	13

Even though the starting material was uneven among the samples, they were analysed for albumin and the biliary cytokeratins CK7 and CK19 [Fig. 4-14]. Albumin and CK19 expression were negative for all the samples in all conditions. CK7 was expressed in all conditions for W3 and W4 but was negative for all conditions in W5. These results correspond to the previous analysis of the colonies in Table 4-3 and 4-4 and the growth factors did not seem to affect the analysed markers.

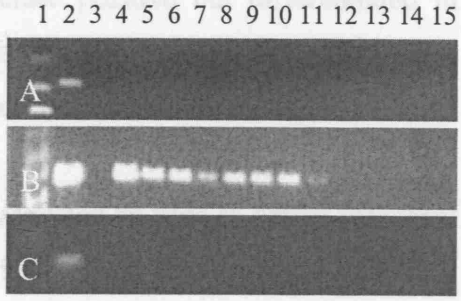


Fig. 4-14 PCR gel showing albumin (A), CK7 (B) and CK19 (C) expression for differentiation experiment of W3, W4 and W5. From left to right – Hyperladder IV (1), positive control (2), template negative control (3), W3 control (4), W3&HGF(5), W3&OSM (6), W3&HGF+OSM (7), W4 control (8), W4&HGF (9), W4&OSM (10), W4&HGF+OSM (11), W5 control (12), W5&HGF (13), W5&OSM (14) and W5&HGF+OSM (15). Note the difference in band brightness and smaller bands in lanes 7 and 11.

Comparing band brightness, CK7 has dim bands for W3&HGF+OSM and W4&HGF+OSM [note: the lanes do not correspond between the gels]. This was an interesting observation, but significant variation was observed in lane density with GAPDH and 18s expression of the samples in Fig. 4-13. Low level of CK7 is correlated with low GAPDH and 18s expression, suggesting it is the low level of starting material that causes dim CK7 bands.

4.6 Discussion

In this chapter it was shown that the npcRTx colony, analysed in *Chapter 3*, did not express telomerase, which might have affected its lifespan in culture. Some stem cells have been found to be telomerase positive, for example embryonic stem cells (Carpenter et al., 2003), whereas other progenitors, such as mesenchymal stem cells, are thought to be telomerase negative (Zimmermann et al., 2003; Banfi et al., 2002). It is also possible that the colony isolated in *Chapter 3* was telomerase positive but differentiated in culture and lost the expression of the enzyme. Unfortunately no earlier sample was tested due to lack of available cells, so no comparison could be made.

Senescence-associated β-galactosidase (SA-β-gal) assay can be used to detect senescent cells in culture (Dimri et al., 1995; Bodnar et al., 1998). Due to the very limited number of culture wells for the npcRTx, this assay could not be performed to investigate the senescence state of the supporting cells or the interesting colony.

In order to extend the lifespan and proliferative capacity of non-parenchymal cell colonies, five different colonies were chosen from two different explant livers. Three of the colonies, isolated from the ALD explant, were successfully transduced with the catalytic sub-unit of telomerase (hTERT). It is unclear whether it is significant that the colonies derived from the fulminant liver explant failed to be transduced. The disease of the patient and other uncontrolled factors might affect the outcome. Moreover, the time of transduction might be critical. The colonies derived from the fulminant explant were cultured for a shorter time (8 days) than those derived from ALD explant.

The three surviving colonies were analysed for hTERT expression. The colonies transduced with hTERT strongly expressed hTERT mRNA, suggesting the transduction worked. Interestingly, the GFP control of W5 also expressed low levels of hTERT, whereas the other two controls were negative. Telomerase inactivation in human somatic cells is mainly due to transcriptional repression and to alternative splicing (Ulaner et al., 1998; Kilian et al., 1997). It has been reported that in some somatic cells the repression of telomerase is not complete. For example Masutomi *et al.* showed that hTERT expression was upregulated in normal, early passage human fibroblasts during the S-phase in the cell cycle (Masutomi et al., 2003). The product was found to be active but transient. These studies highlighted the idea that telomerase and telomere

structure might be dynamically regulated even in normal human cells. hTERT expression in somatic cells is transient, in contrast with the constitutive hTERT expression in most human cancer cell lines. The key to understanding telomerase expression and immortality might lie in the patterns of telomerase expression rather than whether or not a cell expresses it (Masutomi et al., 2003; Counter et al., 1998a).

The growth curves of W3, W4 and W5 showed that the cells did not maintain a constant growth rate. Normal somatic cells have little or no telomerase expression and lose telomeric DNA at every cell division. A point of crisis is reached when the telomeres become critically short. If the cells are, however, transformed with hTERT no such crisis should be reached as the telomeres are maintained with every cell division (Counter et al., 1998a). W5 however, did senesce and W3 and W4 have a decelerating and fluctuating growth rate. This suggests that hTERT may not be sufficient to immortalise these cells and additional mutations in cell cycle regulators may be needed to make them truly immortal. However, additional mutations may cause the cells to transform and lose their putative differentiation capacity.

It is important to note that W3 and W4 have been cultured for a prolonged time (over 600days) compared to W5 (361days), npcRTx [*Chapter 2*] (180days) W1 and W2 (127days). Ideally, the GFP control cells would have been cultured alongside the hTERT transduced cells to investigate the differences in their survival. This was, however, not possible due to limiting time and access to cells.

W3, W4 and W5 were further characterised to express a combination of liver markers, but not specific to either hepatocytes or biliary epithelial cells. The stem cell marker Oct-4 was also observed but, as discussed in *Chapter* 3, it was found to be expressed in HepG2s and whole liver too.

Hepatocytes are known to only express CK8 and CK18, whereas biliary epithelial cells predominantly express CK7 and CK19. However, hepatoblasts are positive for CK8, CK18 and CK19 and oval cells are positive for CK7, CK8, CK18 and CK19 (Omary et al., 2002). W3, W4 and W5 had combinations of markers, which did not correspond to any of the above, e.g. CK7, CK8 and CK18. It was interesting to note that the cells also differed between each other with dissimilar cytokeratin profiles on the protein level, and W5 only expressing GGT on the mRNA level. These differences might give some

insight into the origin of the colonies. For example, W5 had a more biliary-type profile with GGT expression. Moreover, it did not express CK7 and CK19, and only expressed CK8 and CK18 in some cells. Lack of markers is often a sign of an immature phenotype. W4, on the other hand, might have been more hepatocyte-like due to CK8 and CK18 protein expression in most cells of the population. These results were presented as a poster at BASL in 2004 (Laurson et al., 2004).

Differentiation studies with HGF and OSM, however, suggested that the cells did not differentiate into the hepatocyte lineage as expected (Schwartz et al., 2002; Suzuki et al., 2003). No albumin expression was observed and CK7 expression was not down-regulated. Furthermore, no clear and evident morphological changes were observed that would have suggested hepatocyte differentiation.

These analyses highlighted some important points. Firstly, the cell-to-cDNA method of preparing the samples is not ideal as RNA cannot be quantified and the similarity between the samples depends on a similar number of cells at the start. As observed for the differentiation study samples, i.e. when small numbers of cells are prepared, the variations can be large. Only on/off analysis of the markers can be performed and if the starting material is limited, and the marker investigated is expressed at low levels, false negatives can be observed. In order to improve marker analysis and compare levels of transcript, RNA should be quantified and sensitive methods, such as real-time PCR should be utilised.

Another criticism involves the type of immortalisation used in these experiments. Although hTERT transduction is thought to be a good method of immortalizing cells (or prolonging their lifespan) without damaging their genomes, it has been found that hTERT cells might still be making an oncogenic product (Wang et al., 2000). Wang *et al.* showed that TERT driven proliferation seemed to be associated with the activation of the *c-myc* oncogene, and even when TERT was removed (by excising the hTERT retrovirus using Cre recombinase), human mammary epithelial cells had high telomerase activity due to the *c-myc* activation. These results are important to keep in mind when considering how 'normal' hTERT transduced cells are.

Immortalisation of a foetal bipotential primate epithelial liver stem cell has been achieved using retrovirus-mediated transfer of the simian virus 40 large T antigen gene.

The cells were found to grow indefinitely *in vitro* and expressed CK8, 18, 7 and 19, as well as, albumin and AFP. Endogenous telomerase was expressed constitutively. The cells were injected into athymic mice hosts and 50% integrated to liver parenchyma with no tumorgenicity (Allain et al., 2002).

4.6.1 Conclusion

The results in this chapter suggested different origins for the three characterised colonies due to their differential expression of liver markers. Furthermore, it has been identified that different explant livers as starting material generate different outcomes. The colony isolated by Selden *et al.* (Selden et al., 2003) was isolated from a sub-fulminant liver explant, the npcRTx colony discussed in *Chapter 3* was isolated from a failing/rejected transplant (Laurson et al., 2003) and the colonies discussed in this chapter were from an ALD explant (Laurson et al., 2004). Among these successes, a large proportion of non-parenchymal cells cultured from different explants (sometimes with the same disease aetiologies mentioned above) do not yield putative progenitor colonies. For practical isolation of putative stem cells, it is crucial to be able to identify these cells from the starting population, before any culture. The next chapters will tackle issues around the analysis and sorting of the starting population of non-parenchymal cells with different markers associated with stem cells and liver progenitors in order to obtain a homogenous population of cells which, putatively, have a high proliferative potential.

Chapter 5

Flow cytometric analysis of the isolated non-parenchymal cells

5.1 Introduction

Data from flow cytometric (FCM) analysis of different cell marker expression by non-parenchymal cells in various liver diseases will be presented in this chapter. As the aim of this work was to isolate populations enriched for liver cell progenitors, markers relevant to progenitor cell populations were studied. Some differences between marker expression by non-parenchymal cells isolated from normal (BS) and from explant liver were determined. The technical problems involved in analysing non-parenchymal cells by flow cytometry and the issues involved in their analysis are also discussed.

5.1.1 Choosing markers

For practical use of liver stem cells it is essential to distinguish and separate them from the heterogeneous population of cells derived from the collagenase liver perfusion. A number of putative stem cell markers were therefore identified. Surface markers are particularly useful as they can be used to pull out the desired cells using cell sorting methods, whilst maintaining viability.

The introduction is divided into three parts, each focusing on a different set of markers that could be used for liver progenitor enrichment. 5.1.2 introduces the ABC-transporter protein ABCG2, 5.1.3 discusses c-met and CD49f and lastly, 5.1.4 deals with the haematopoietic markers CD117 and CD133.

5.1.2 ABCG2 as a marker for stem cells

5.1.4.1 The side population, Hoechst dye efflux and ABCG2

A small population of cells effluxing Hoechst 33342 dye was first identified by flow cytometry in a murine bone marrow sample (Goodell et al., 1996). This population was termed 'side population' (SP) and these cells were found to have phenotypic markers of haematopoietic stem cells and a 1000-fold enrichment for repopulation activity. The

efflux capacity was blocked in the presence of verapamil, a calcium channel blocking drug (Goodell et al., 1996).

Goodell *et al.* later identified SP cells in human, pig and rhesus macaques' bone marrow (Goodell et al., 1997). The SP cell is a rare event, only 0.05% (0.03-0.09%) of cells in human bone marrow efflux the dye and 0.1% in cord blood (0.15-0.07%) (Goodell et al., 1997).

Scharenberg *et al.* and Zhou *et al.* investigated the pump involved with the Hoechst efflux. Scharenberg *et al.* determined ABCG2 [MXR1, BCRP, ABCP] (ATP-binding cassette (ABC) subfamily G member 2) to be the molecular determinant of the SP in the lung carcinoma cell line A549 (Scharenberg et al., 2002). Moreover, a SP phenotype was acquired by transient expression of ABCG2 in human embryonic kidney cells (HEK293) (Scharenberg et al., 2002) and Saos-2 cells (Zhou et al., 2001). High levels of ABCG2 were found in SP cells compared with other ABC transporters in both human and murine bone marrow (Scharenberg et al., 2002; Zhou et al., 2001).

ABCG2 and other ATP-binding cassette (ABC) transporters (e.g. P-glycoprotein [MDR1/ABCB1]) are transmembrane proteins involved in energy-dependent transport of substrates across membranes. They are found in cancers that are intrinsically resistant to conventional chemotherapy and the pump proteins are therefore known as multidrug resistance proteins (Ejendal and Hrycyna, 2002; Scharenberg et al., 2002). The multi-drug resistance proteins exhibit overlapping substrate specificities. They have the ability to efflux broad ranges of lipophilic molecules including Hoechst 33342 (Scharenberg et al., 2002).

In stem cells, ABCG2 is thought to serve a protective function by preventing toxins from entering cells as well as potentially playing a role in regulating stem cell differentiation and growth by excluding small lipophilic molecules such as steroids (Ejendal and Hrycyna, 2002; Scharenberg et al., 2002). ABCG2 is abundantly expressed in placenta, liver, intestine as well as stem cells (Ejendal and Hrycyna, 2002; Scharenberg et al., 2002).

5.1.2.1 SP and ABCG2 expression in haematopoietic stem cells

In bone marrow derived haematopoietic cells, human and rhesus SP cells were found to be mainly $CD34^{low}$ and only 1-2% were $CD34^{+}$(Goodell et al., 1997). Furthermore, efflux activity was found to correlate with the maturation state of the haematopoietic cells, with highest efflux activity in the most primitive stem cells. (Goodell et al., 1997). Similarly, ABCG2 was found to be expressed at relatively high levels in stem cells and expression was found to drop sharply in committed progenitors in both human and mice (Scharenberg et al., 2002; Zhou et al., 2001). Enforced ABCG2 expression in murine bone marrow cells inhibited haematopoietic development (Zhou et al., 2001).

In fact, P-glycoprotein has been associated with $CD34^{+}$ haematopoietic cells and Rhodamine123 efflux (Chaudhary and Roninson, 1991; Bunting, 2002; Kim et al., 2002). It is therefore thought that the more primitive haematopoietic stem cells ($CD34^{neg}$) express ABCG2, and the more committed progenitors ($CD34^{+}$) express P-glycoprotein that will efflux Rhodamine123. ABCG2 does not efflux Rhodamine123 (Bunting, 2002; Kim et al., 2002).

5.1.2.2 SP and ABCG2 expression in liver and other stem cells

SP cells have been investigated in mouse ES cells, but the non-SP fraction was also found to contain pluripotent cells (Zhou et al., 2001).

In adult tissue Asakura *et al.* (Asakura and Rudnicki, 2002) showed that SP cells could be isolated from mouse skeletal muscle (3.1% of total nucleated cells), heart (9.1%), brain (15.1%), spleen (0.96%), liver (4.3%), kidney (5.8%), lung (0.98%), small intestine (8.6%) and bone marrow (0.79%). Haematopoietic progenitor colony formation assays were performed on the SP populations isolated from the different organs. All gave rise to colonies at a higher frequency than the respective non-SP populations. The haematopoietic marker CD45 was observed in SP cells at different proportions in the different organs. Of the SP cells isolated from the liver only 39% were $CD45^{+}$ (Asakura and Rudnicki, 2002). However, not all SP cells isolated from adult tissue are stem cells. Triel *et al.* investigated human and mouse epidermis SP cells and found them to lack stem cell characteristics (Triel et al., 2004).

Uchida *et al.* isolated SP cells from adult mouse liver (Uchida et al., 2002). Around 1-2% of the liver cells had a SP phenotype. The cells were found to be heterogeneous

even within $CD45^+$ cells (around 20-25% of the SP cells). However, more than 90% were found to be long-term culture-initiating cells or in vivo repopulating cells. Mice lacking P-glycoprotein (Pgp-/-) were found to have similar SP populations. In conclusion, the adult murine liver was found to contain a spectrum of haematopoietic cells that were phenotypically and functionally similar to those in the bone marrow (Uchida et al., 2002).

Shimano *et al.* used a rat model with partial hepatectomy and AAF (2-acetylaminofluorene) to look at the SP population in injured liver (Shimano et al., 2003). Oval cells were detected at day 7, but not on day 0. Similarly, an SP population (2% from total non-parenchymal cells) was also seen at day 7, but not at day 0. A comparable correlation was also seen when observing mRNA expression. At day 7 ABCG2 was strongly expressed in whole liver, whereas it was barely detectable at day 0. However, the expression thereafter gradually decreased. The cells expressing ABCG2 were shown by *in situ* hybridization analysis to be associated with oval cells that were present in the periportal field and the surrounding parenchyma (Shimano et al., 2003).

Wulf *et al.*, on the other hand, used a murine model to look at liver SP cell contribution to liver regeneration (Wulf et al., 2003). The SP cells isolated from whole liver accounted for 1% of the mononuclear cells and were small, blast-like cells with agranular cytoplasm. Of these cells 75.5% expressed CD45, whereas the cells with the highest efflux potential were enriched in $CD45^{neg}$ cells. The SP cells also contained distinct sub-populations of CD34, CD117, Sca-1 and Thy-1 positive cells. When the hepatic SP cells were transplanted into the livers of DDC-treated mice, they survived, expanded and contributed to liver regeneration. Moreover, bone marrow SP cells were also able to be involved in the regeneration of the liver (Wulf et al., 2003).

In *vitro studies* of the liver SP cells were investigated in haematopoietic stem cell supporting methylcellulose media. Seven (±2.9) colonies were derived from $2x10^4$ cells (Wulf et al., 2003). Only the SP cells showed growth in methylcellulose, compared with the non-SP cells. Unsorted hepatic progenitor cells rarely formed colonies (3 of $2x10^5$ liver cells plated). In cultures supplemented with hepatocyte growth stimulants (EGF, TGF-α and nicotamidine) there was no obvious growth. Even when they were added after haematopoietic progenitor stimulants, only slight proliferation was seen. This

suggested that hepatocyte growth factors had an inhibitory effect on early progenitor growth (Wulf et al., 2003).

Alison questions the importance of a SP compartment in the liver in his review based on other functions of the ABC transporters (Alison, 2003). ABCG2 is expressed in bile duct canalicular in normal liver (Doyle and Ross, 2003) and Ros *et al.* have shown that a variety of different ABC transporters are upregulated during liver disease (Ros et al., 2003a; Ros et al., 2003b). The transporters play an important role in secretory and protective functions. Ros *et al.* investigated ABC transporter pump expression in human liver disease and rat models (ABCB and ABCC subfamilies) and found that ABCB1 (efflux pump for hydrophobic compounds) ABCC1 and ABCC3 (transporters of amphilic anionic conjugates into bile) were all upregulated in reactive bile ductules/progenitor cells. The upregulation of these proteins offers protection from toxic bile constituents and maybe results in these cells becoming resistant to oxidative stress. The question that Alison raises in his review is that the expression of ABC transporters in the ductules might only reflect the protection mechanism within the biliary tree against the toxic bile constituents and it is unlikely that all reactive ductular cells are stem or progenitor cells. However, he also suggests that a more specific SP phenotype can be found that would characterize SP cells with stem cell properties (Alison, 2003).

5.1.3 CD49f and c-met as markers for stem cells

Suzuki *et al.* used markers CD49f and c-met to isolate putative stem cells from foetal mouse liver (Suzuki et al., 2000a). C-met is the receptor for HGF and HGF is involved both in hepatic maturation and in activating quiescent hepatocytes to proliferate during liver injury (Kamiya et al., 2001; Suzuki et al., 2003; Stuart et al., 2000). CD49f (α6 integrin/VLA-6α) forms dimers with β1 (CD29) and β4 (CD104) sub-units and these dimers are receptors for laminin. CD49f is also involved in foetal liver development and is upregulated in human liver disease (Couvelard et al., 1998; Nejjari et al., 2001).

Firstly, Suzuki *et al.* used flow cytometry (FCM) to sort $CD45^{neg}TER119^{neg}$ cells further by expression of integrins α6 (CD49f) and β1 (CD29). The $CD49f^{+}CD29^{+}$ fraction was further subdivided into $CD117^{+}$ and $CD117^{neg}$ populations. It was found that $CD49f^{+}CD29^{+}CD117^{neg}$ sorted cells were enriched 34.9-fold with hepatic colony-forming units in culture [H-CFU-C] (single cells proliferate to form clusters of more

than a hundred cells) compared with total foetal liver (Suzuki et al., 2000a). Moreover, albumin and CK19 expression was seen after five days in culture. The cultures expressed c-met and HGF was required for culture. The $CD45^{neg}TER119^{neg}$ $CD49f^{+}CD29^{+}$ $CD117^{neg}$ cells were also transplanted into recipient mouse spleens and the cells were found to migrate to the recipient liver parenchyma and differentiate into hepatocytes (Suzuki et al., 2000a).

The $CD45^{neg}TER119^{neg}CD49f^{+}CD29^{+}CD117^{neg}$ cells were further examined for the expression of c-met (Suzuki et al., 2002). The $CD45^{neg}TER119^{neg}CD117^{neg}$ cells were further subdivided into different expression patterns of CD49f (+/high, +/low and negative) and $c\text{-}met^{+}$ and $c\text{-}met^{neg}$. The $CD49f^{+/low}c\text{-}met^{+}$ fraction was found to be 560-fold enriched with H-CFU-C compared to total foetal liver. Some of the H-CFU-C gained expression of CD117, CD34 and Thy-1, all putative markers of adult liver progenitors/stem cell (Suzuki et al., 2002).

In *in vivo* studies, the $c\text{-}met^{+}CD45^{neg}$ cells were transplanted into retrorsine-treated adult rats with 2/3 hepatectomy. At 9 months post transplant, donor derived cells had formed colonies that included mature hepatocytes expressing albumin and containing abundant glycogen in their cytoplasm. $C\text{-}met^{neg}$ or $CD45^{+}$ cells did not repopulate the livers (Suzuki et al., 2004).

C-met expression has also been found on human cord blood $CD34^{+}$ progenitor cells (Goff et al., 1996). HGF acted as a synergist *in vitro* and enhanced colony formation of $CD34^{+}$ progenitors (Goff et al., 1996).

In our laboratory, c-met expression was observed in putative progenitors isolated by Selden *et al.* (Selden et al., 2003), in the cell colony npcRTx isolated in *Chapter 3* and in hTERT transfected non-parenchymal cell colonies W3, W4 and W5 analysed in *Chapter 4*.

5.1.3.1 Integrins in liver development and disease

Integrins have been established to be important in liver development both in the mouse and humans. In foetal mice it was found that the co-expression of $\alpha6\beta1$ ($CD49f^{+}CD29^{+}$) dramatically decreased as the foetal liver developed (Suzuki et al., 2000a).

Integrins are likely to play an important role in the differentiation of epithelial and endothelial cell populations of the liver (Couvelard et al., 1998). During human liver organogenesis, hepatoblasts express β1, α1, α5, α6 and α9 integrin chains (including the α1β1, α5β1, α6β1 and α9β1 dimers). Foetal hepatocytes (8^{th} week of gestation) initially retained the same combination of integrins, but the expression levels progressively decreased. After 15 weeks of gestation, the expression of β1, α1, α5 and α9 reached levels comparable to adult hepatocytes. Alpha 6 expression, however, became undetectable after 30 weeks of gestation. As compared with hepatoblasts, intrahepatic biliary epithelial cells were characterised by progressive loss of α1 and marked induction of α6 and *de novo* acquisition of β4, α2 and α3 (increased expression of α6β1, loss of α1β1) (Couvelard et al., 1998). The integrin dimer α6β1 therefore seems to play a critical role in liver development.

Integrin upregulation has also been seen in chronic liver disease both on hepatocytes and sinusoidal lining cells. Nejjari *et al.* studied integrin expression in chronic hepatitis C patients (Nejjari et al., 2001). Over-expression of integrin receptors only weakly detectable on normal hepatocytes (α1β1 and α5β1) was detected, as well as, the induction of receptors normally undetectable on hepatocytes including α6β1 and α2β1.

Hoppo *et al.* isolated $CD49f^+$ mouse foetal hepatic progenitor cells (Hoppo et al., 2004). The cells expressed AFP, albumin and CK19. The cells required co-culturing with $Thy1^+$ cells (which were negative for the above markers) to mature morphologically, store glycogen and increase expression of TAT and tryptophan oxygenase.

5.1.4 CD117 and CD133 as markers for stem cells

5.1.4.1 CD117 and CD133 as haematopoietic stem cell markers

CD117 and CD133 were first described in haematopoietic cells as stem cell markers. CD117 (c-kit) was identified as a 145kD cell surface receptor tyrosine kinase (Yarden et al., 1987) and is expressed on a variety of cell types, including haematopoietic progenitor cells, mast cells, melanocytes, germ cells and gastrointestinal pacemaker cells (Akin and Metcalfe, 2004). CD117 is the receptor of stem cell factor (SCF) (Broudy et al., 1992).

19-51% of $CD34^+$ bone marrow progenitor cells co-express CD117 (Strobl et al., 1992). Ratajczak *et al.* investigated sorted haematopoietic stem cells subsets $CD34^+CD117^+$, $CD34^+CD117^{neg}$ and $CD34^{neg}CD117^{neg}$ by transplanting them into an immunodeficient SCID mice model. Only $CD34^+CD117^+$ cells were able to establish haematopoietic chimerism in these animals (Ratajczak et al., 1999). Interestingly, it was also found that the fluorochrome used influenced the detection sensitivity of CD117 expressing cells. Cy5 was more sensitive than PE, which was more sensitive than FITC (Ratajczak et al., 1999). CD117 is downregulated from the surface of the haematopoietic progenitors as they differentiate into different lineages. In mast cells, however, CD117 is retained (Akin and Metcalfe, 2004).

CD133 was described as a novel stem cell glycoprotein antigen expressed on the $CD34^+$ haematopoietic stem cells derived from human peripheral blood, bone marrow, cord blood and foetal liver and bone marrow (Yin et al., 1997; Miraglia et al., 1997). The 5-transmembrane molecule has a molecular weight of 120kD and no known function or ligand. CD133 was expressed on 20-60% of $CD34^+$ cells and a few other tissues such as retina, pancreatic islet cells and placenta (Yin et al., 1997; Miraglia et al., 1997).

Kopari *et al.* compared the ex vivo expansion of $CD34^+$ and $CD133^+$ haematopoietic stem cells isolated from cord blood and found that the $CD133^+$ cells could be expanded in the same manner as $CD34^+$ cells with similar multilineage capacity (Kobari et al., 2001). Moreover, De Wynter *et al.* found that $CD34^+CD133^+$ cells isolated from human bone marrow and cord blood had 10-100-fold higher generation of progenitor cells than $CD34^+CD133^{neg}$ cells. Furthermore, only $CD34^+CD133^+$ were capable of repopulating NOD-SCID mice (de Wynter et al., 1998).

In fact, some studies suggest that CD133 might be an earlier marker than CD34 for haematopoietic stem cells. Gallacher *et al.* investigated $CD34^{neg}CD38^{neg}Lin^{neg}$ sub-fractions from human cord blood and found that $CD133^+CD7^{neg}$ cells (0.2% of the $CD34^{neg}CD38^{neg}Lin^{neg}$ cells) had progenitor activity similar to $CD34^+$ cells. The $CD133^+CD7^{neg}$ cells could also give rise to $CD34^+$ cells *in vitro* and were capable of engrafting NOD-SCID mice (Gallacher et al., 2000).

Furthermore, Kuci *et al.* found that $CD133^+$ cells isolated from human mobilised peripheral blood and cultured for 3-5 weeks, gave rise to $CD133^+CD34^{neg}$ cells

incapable of generating haematopoietic colonies, but with long-term multilineage engraftment in NOD-SCID mice at a higher engraftment than freshly isolated $CD34^+$ cells (Kuci et al., 2003).

Double positive ($CD133^+CD117^+$) have been observed in cord blood. These cells lost expression of both surface markers *in vitro* as they differentiated (Ruzicka et al., 2004).

5.1.4.2 CD117 and CD133 in liver

The identification of $CD117^+$ cells in liver is reported by several different groups and immunohistological examination of liver sections has been carried out both in diseased and normal liver. Some studies suggest that $CD117^+$ cells may represent the hepatic progenitor cell population.

Fujio *et al.* demonstrated in rat CD117 and SCF expression in the ductular cells around the portal vein during the late embryonic stage of the liver and in adult liver in both bile ducts and ductules (Fujio et al., 1996). Partial hepatectomy was used together with acetylaminofluorene to activate the stem cell compartment, and SCF and CD117 were seen in the infiltrating oval cell population but absent in newly formed basophilic hepatocytes. It was concluded that SCF and CD117 were not involved in proliferation of hepatocytes but were present in oval cells and the expression was turned off when the oval cells differentiated (Fujio et al., 1996).

$CD117^+$cells have been found in portal tracts (Baumann et al., 1999), the canals of Hering and the intralobular bile ducts of normal human liver (Theise et al., 1999). In cirrhotic tissue the number of $CD117^+$ cells was increased, although mRNA levels remained relatively consistent (Baumann et al., 1999). However, in massive hepatic necrosis/fulminant liver failure $CD117^+$ cells were also found integrated in bile ducts and higher mRNA levels were observed in some cases (Theise et al., 1999; Baumann et al., 1999). Although some of these cells were found to express mast cell, leukocyte and haematopoietic markers, some $CD117^+$ cells lacked such markers (Baumann et al., 1999).

A further study by Seki *et al.* studied the livers of patients with fulminant massive necrosis and found CK14 positive cells that also expressed CD117, flt-3 and CK19 but

were negative for albumin, Ki-67 and CD34 (Seki et al., 2003).The cells were found in a small number of cells lining biliary ductile-like structures.

Craig *et al.* have investigated hepatopoiesis in massive hepatic necrosis. Hepatopoiesis was found to take place in the residual portal tracts and ductular reactions where there also is a lymphoid infiltrate. Non-hepatocytic, non-biliary, small, round or oval cells with a diameter of 7-11μm with scanty cytoplasm were identified. $CD133^+$ cells, that also express CD117, were seen in ductules and by occasional solitary lymphoid blast-like cells. Similar circulating cells were present in central veins and sinusoids. $CD117^+CD133^+$ cells did not express CD34, CD45 or tryptase (Craig et al., 2004b; Craig et al., 2004a).

In vitro studies have shown that cells isolated using CD117 and CD34 from human cirrhotic and normal liver were found to be putative liver progenitor cells (Crosby et al., 2001). The cells were cultured for seven days in biliary epithelia supporting media and found to differentiate into the biliary epithelial cell lineage.

These studies together suggest there are cells expressing CD117 and CD133 found in the liver. Their specific role in liver regeneration and origin (bone marrow *versus* liver) is not clear, but they are good candidates as putative liver stem cells.

5.2 Hypotheses

The hypotheses of this chapter were:

1) In human explant liver, populations of potential stem cells may be identified by flow cytometric analysis using a combination of surface markers.
2) The liver explant aetiology most enriched in specific sub-population of putative stem cells can be identified.

5.3 Aims

The aims of this chapter were to:

1) Use FCM analysis to study different explant liver samples for the presence of cell populations expressing CD117/CD133, C-met/CD49f and ABCG2.
2) Compare the different liver disease aetiologies for the proportion of cells expressing a specific marker or combination of markers in order to identify differences between different disease states in respect of putative stem cells.

5.4 Methods

5.4.1 Starting material - Non-parenchymal liver samples

The non-parenchymal cells were isolated, frozen and thawed from human explant livers and resected tissue as described in *Chapter 2 General Methods*. The samples used in each experiment are detailed at the beginning of each section.

5.4.2 Positive controls

Positive controls were used to set up antibody titres and for other control experiments.

5.4.2.1 Weri-Rb-1

Weri-Rb-1 cells express CD133 and were used as a control for experiments assessing this antigen.

5.4.2.2 MO7e

MO7e cells express CD117 and were used as a control for experiments observing this antigen.

5.4.2.3 HepG2

HepG2 cells express c-met and CD49f and were used as a control for experiments observing these antigens.

5.4.2.4 HT-29

HT-29 cells express ABCG2 (Imai et al., 2002) and HEA125 and were used as a control for experiments assessing these antigens.

5.4.2.5 VLB/CEM

CEM/VLB cells express P-glycoprotein and were used as a control for the functional calcein-AM assay (Beck et al., 1988). These controls were prepared and provided by Kanagasabai Ganeshaguru, Department of Haematology, Royal Free and University College Medical School.

5.4.2.6 Peripheral blood monocytes (PBMCs)

The cells were used as positive controls for markers CD34, CD45 and CD49f. For preparation of PBMC, please refer to *Chapter 2 General Methods*.

5.4.3 Negative controls

Isotype-matched controls were used to confirm low non-specific binding of the antibodies used. Details of the negative controls used are found in each experimental section. For more information on the use of isotype-matched controls please refer to section 5.4.6.2.

5.4.4 Preparing the cells for FCM analysis

The cells were stained with fluorochrome-linked antigen-specific antibodies or using secondary antibodies with linked fluorochromes. For specific incubation time, volume and temperature please refer to *Chapter 2 General Methods*. Table 5-1 summarises the antibodies used in this chapter.

Table 5-1 The antibodies used for FCM analysis. The antibodies are 'conjugated' if the primary antibody has a fluorochrome tag. Otherwise an antigen specific primary is used together with a fluorochrome-linked secondary antibody.

Antigen	Clone	Fluorochrome used	Conjugated?
CD117	95C3	PE-Cy5 [PC5]	yes
CD133/1	AC133	Phychoerythrin [PE]	yes
CD45	J33	Fluorescein isothiocyanate [FITC]	yes
CD34	581	Allophycocyanin [APC]	yes
c-met	DL-21	FITC	no
c-met	DO-24	FITC	no
c-met	poly	AlexaFluor 488 and PE	no
CD49f	MP4F10	FITC and PE	no
ABCG2	5D3	PE	yes
HEA125	HEA125	FITC	no

5.4.5 FCM analysis with BD Bioscience FCMCalibur

5.4.5.1 Acquisition template

Normally around 20 000 events are acquired from a sample. Low numbers of positive cells were expected from the liver samples, so 20 000 events inside a 'cells of interest' gate were acquired. The 'cells of interest' gate is set up in the forward scatter (FSC) versus side scatter (SSC) dot plot shown in Fig. 5-1. SSC gives an indication of cell granularity and FSC gives an indication of cell size. The gate was selected based on the morphology (agranular) and size (small) of putative stem cells. Debris (very low SSC

and FSC) and large clumps of cells (very high SSC and FSC) are also excluded from this gate.

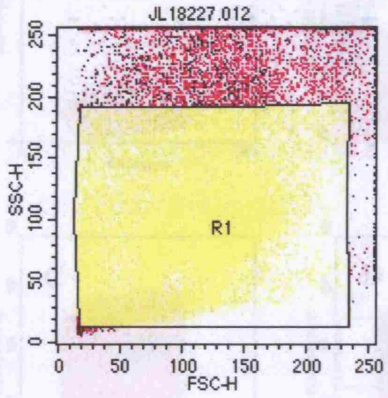


Fig. 5-1 Showing the 'cells of interest' gate used for data acquisition. The gate selects for putative stem cells which are agranular and small in size.

5.4.5.1.1 CD117/CD133 acquisition gate

The number of CD117/CD133 double positive cells were thought to be extremely low, so a more specific 'cells of interest' gate and an additional acquisition gate was set up [Fig. 5-2]. To create the additional gate, the CD45 antibody was used. In blood samples CD45 is often used to indicate nucleated haematopoietic cells because most cells express this antigen at some level. To choose an appropriate CD45 gate, which would help to reduce background by excluding cell debris, the $CD117^{+}$ cells were back-gated onto the CD45 versus SSC dot-plot [Fig. 5-3]. The dot-plot showed what level of CD45 expression the $CD117^{+}$ cells had. Most cells were found to be $CD45^{low}$. A gate was drawn in this area as shown in Fig. 5-2. For acquisition, only cells that were found both in the live gate and $CD45^{low}$ gate were saved (250 000 events acquired).

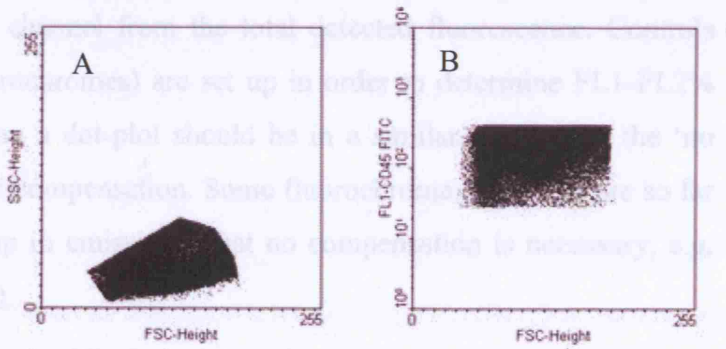


Fig. 5-2 Showing 'cells of interest' gate (A) and additional acquisition gate (B) for CD117/CD133 experiments. Gate in (B) was determined using back-gating of CD117 cells to the CD45 dot-plot [Fig. 5-3]. The $CD117^{+}$ cells were found to be $CD45^{low}$.

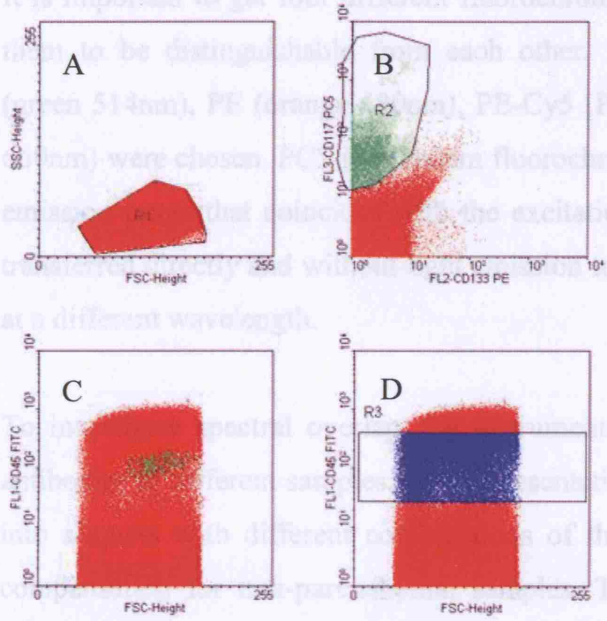


Fig. 5-3 CD117 back-gating for a CD45 acquisition gate. (A) 'cells of interest gate' in SSC versus FSC dot-plot. (B) cells inside cells of interest gate are analysed on CD117 versus CD133 dot-plot and $CD117^{+}$ cells are gated in R2. (C) R2 cells shown in green on CD45 versus FSC dot-plot and (D) $CD45^{low}$, where $CD117^{+}$ cells are found, are gated for acquisition.

5.4.5.2 Compensation

5.4.5.2.1 Compensation for two colour analysis

Compensation is required in multi-colour analysis due to spectral overlap between the emission spectra of fluorochromes (Ortolani, 2004). For example, if cells are labelled only with FITC-conjugated antibody, upon analysis a small proportion of the FITC emission will appear in the PE channel and be detected as positive events (FL2 in this case). The intensity of the faint fluorescence is dependent on the amount of overlap of the emission. Fluorescence compensation can be used to remove the apparent overlap at the risk of loosing weakly positive PE signals. This is done by removing the amount of leaked fluorescence from that channel from the total detected fluorescence. Controls (cells labelled with single fluorochromes) are set up in order to determine FL1-FL2% and FL2-FL1%. The clusters on a dot-plot should be in a similar position to the 'no antibody' negative control after compensation. Some fluorochrome emissions are so far from each other, i.e. no overlap in emissions, that no compensation is necessary, e.g. APC and FITC (Ortolani, 2004).

5.4.5.2.2 Compensation for four colour analysis – CD117/CD133 experiments

The choice of fluorochromes, compensation and controls are very important for four colour analysis. Four colour settings and compensations were designed by Dr Mark Lowdell, Department of Haematology, Royal Free and University College Medical School.

It is important to get four different fluorochromes to emit at different wavelengths for them to be distinguishable from each other. For CD117/CD133 experiments FITC (green 514nm), PE (orange 580nm), PE-Cy5 [PC5] (far red 670nm) and APC (far red 660nm) were chosen. PC5 is a tandem fluorochrome where the first molecule PE has an emission range that coincides with the excitation range of the Cy5. The excitation is transferred directly and without light emission to the second molecule which then emits at a different wavelength.

To investigate spectral overlap, the instruments were set up using a combination of antibodies in different samples. One representative sample, a PBC explant, was divided into aliquots with different combinations of the antibodies to set up the acquisition compensation for non-parenchymal samples. The settings derived from this control sample were used for the following experiments.

5.4.6 Data Analysis

5.4.6.1 Histograms versus dot-plots and cluster analysis

It should always be noted that FCM data are comparable to data derived by looking down a microscope. It does not provide fixed numbers but the decision made by the observer is the outcome of the analysis (Lowdell, 2003).

The traditional way of looking at FCM data has been by histograms. This analysis provides information on the sample by looking at one characteristic at a time (SSC/FSC/FL-channel) against the number of cells. The histograms are used together with isotype-matched controls. Using the isotype-matched control a line (gate) is drawn to determine positive events, i.e. cells not overlapping the negative control.

Cluster analysis (introduced in the 1980's), on the other hand, will look at two parameters on a dot-plot. The dot-plot will also provide information on the number of cell in any one area by the density of the dots (Lowdell, 2001). Fig. 5-4 shows an example of $CD34^+$ cells in a sample of peripheral blood monocytes both on a histogram and on a dot-plot. The positive cells create a fluorescent cluster of cells seen clearly in a dot-plot, but easily missed on a histogram. Most biological samples have an internal negative control present in the samples. For example, when staining for CD34, the majority of the cells will be negative and a small population of cells will be positive.

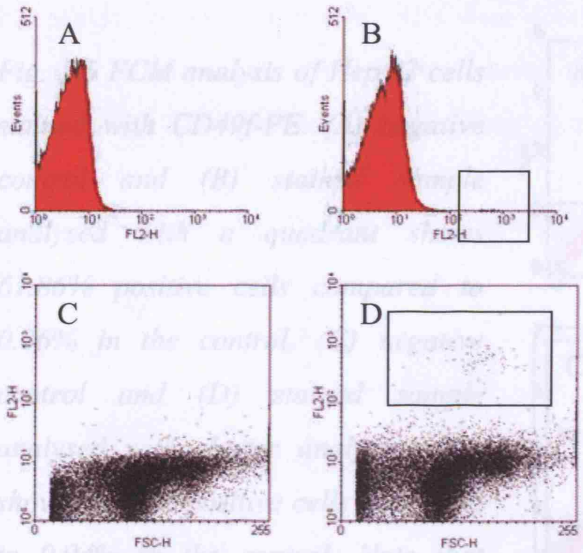


Fig. 5-4 FCM analysis of CD34 stained PBMCs. (A) unstained sample and (B) stained sample represented as histograms. The same (C) unstained sample and (D) stained sample represented as dot-plots. Note the small cluster of cells in (D) and the small peak of events in (B) inside the boxes.

In some cases cluster analysis is difficult to apply. In cell lines, for example, it is possible that negative cells do not exist, and therefore no cell clusters are present. The data from such experiments need to be carefully analysed due to the nature of marker expression. If the control and stained samples overlap, it would make sense to interpret that the cells overlapping the negative control are negative, whereas the cells that do not overlap are positive. However, it is important to realise that cells that move from the lower range in the negative control need to stain 10 times more to move over the line of what is considered positive, than cells that are found just under the line (Fig. 5-6 shows an example of such a gate). In the case where the distribution of the antigen is expressed by most of the cells at a similar level and where it overlaps with the negative control, it might be more sensible to analyse the data in terms of degree of fluorescence rather than percentage of positive cells (Lowdell, 2001). For positive control experiments using cell lines, it is often enough to be able to conclude that the cells stained with the antibody have a clear shift and the median fluorescence or exact percentage of positive cells is unimportant.

Another analysis method that sometimes is difficult to use in cluster analysis is quadrants. If the cells are easily resolved along straight lines it works well, but Fig. 5-5 shows quadrant analysis of a sample which does not go well with the data and underestimates the number of positive cells. A better approach to this sort of data is

achieved using gates. The same gate should be kept for the control and stained samples, but should be redrawn for each biological sample (Lowdell, 2003).

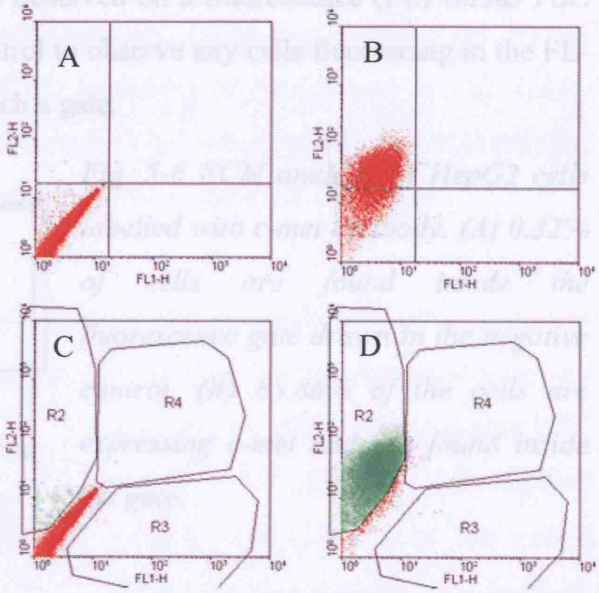


Fig. 5-5 FCM analysis of HepG2 cells stained with CD49f-PE. (A) negative control and (B) stained sample analysed with a quadrant shows 61.86% positive cells compared to 0.06% in the control. (C) negative control and (D) stained sample analysed with cluster analysis gates shows 94.56% positive cells compared to 0.94% in the control. Note that 31.82% less cells are considered positive if using quadrants.

5.4.6.2 Isotype-matched controls

Until 10 years ago an isotype-matched control monoclonal antibody conjugated with the same fluorochrome as the antibody of interest was usually used when analysing samples for phenotypes. This was used to give an idea of the background fluorescence created by non-specific binding of an antibody. However, a good isotype-matched control is difficult to obtain as it should have the same fluorochrome:protein ratio as the test antibody, the same proportion of monovalent and divalent antibody molecules and similar non-specific binding kinetics. Therefore, when working with biological material with internal negative controls and using cluster analysis, an isotype-matched control is not necessary (Lowdell, 2001). If, however, staining is gradual and no clear negative and positive populations are observed, it is useful to have an idea of the non-specific binding of an isotype-matched control, although the limitations of this control should be taken into consideration.

5.4.6.3 Percentage of positive cells

5.4.6.3.1 Single colour analysis

For single colour analysis the cells were observed on a fluorescence (FL) *versus* FSC dot-plot. A gate was drawn using the control to observe any cells fluorescing in the FL-channel. Fig. 5-6 shows an example of such a gate.

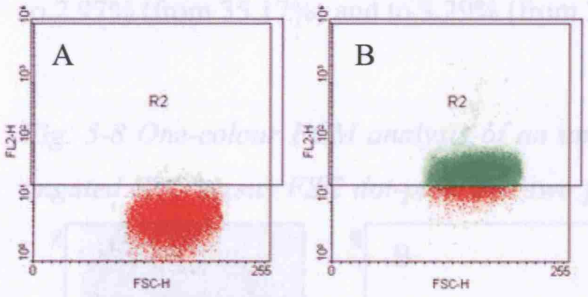


Fig. 5-6 FCM analysis of HepG2 cells labelled with c-met antibody. (A) 0.32% of cells are found inside the fluorescence gate drawn in the negative control. (B) 85.86% of the cells are expressing c-met and are found inside the gate.

5.4.6.3.2 Two-colour analysis

In order to observe two different antigens at once, the fluorochromes were plotted against each other, i.e. FL2 versus FL1. Three gates were drawn in order to observe double positive cells and cells which were positive for one marker and negative for the other. Fig. 5-7 shows an example of gates drawn to analyse the expression of two markers.

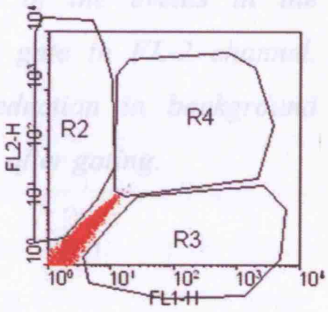


Fig. 5-7 A two-colour analysis dot-plot. The FL2 (PE) channel has been plotted against FL1 (AlexaFluor 488). R2 gates all events that will be considered positive for PE but negative for FITC. R3 gates for all events considered positive for FITC but negative for PE. Double positive events gated in R4.

5.4.6.3.3 Four-colour analysis

In order to analyse CD117/CD133 double positive cells, four-colour analysis was used. CD45-FITC was used to set up the acquisition gate. CD117-PC5 and CD133-PE were used to identify the cells (as for Fig. 5-7) and CD34-APC was used to investigate if the double positive cells were haematopoietic in origin.

5.4.6.4 Background fluorescence – Gating for cell populations

High background fluorescence was experienced when analysing non-parenchymal liver samples. To reduce the fluorescence of the unlabelled samples, two clear cell populations from the SSC *versus* FSC dot-plots were gated and used for any further analysis. Fig. 5-8 shows an example of the reduction of background in one colour analysis of an unstained non-parenchymal sample. The background in FL1 was reduced to 2.97% (from 35.17%) and to 3.29% (from 35.14%) in the in FL2 channel.

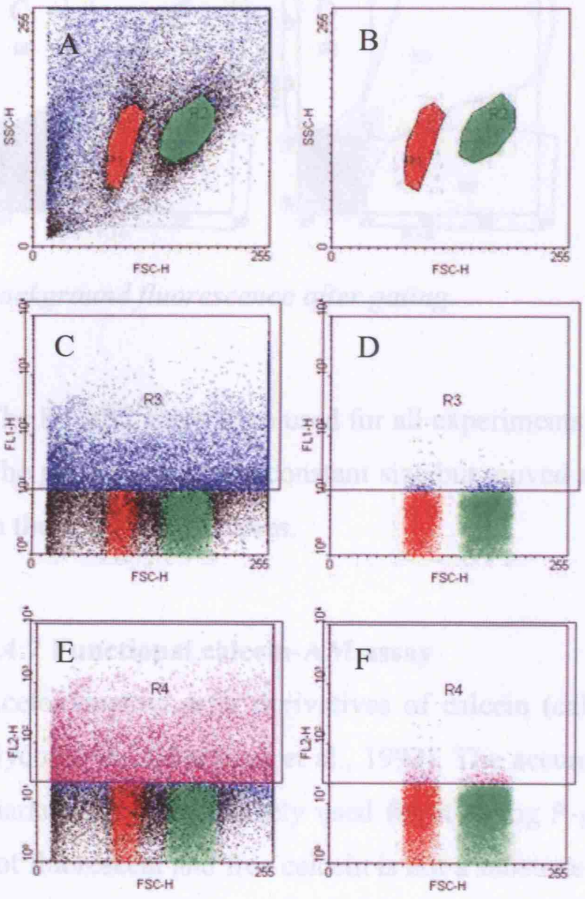


Fig. 5-8 One-colour FCM analysis of an unstained non-parenchymal cell sample. (A) ungated SSC versus FSC dot-plot. The two populations of interest can be seen in red and green. (B) R1orR2 gated SSC versus FSC dot-plot. (C) ungated sample with 35.17% of the events inside the fluorescence gate in FL-1. (D) Sample gated on R1orR2 with 2.97% of the events inside the fluorescence gate in FL-1. (E) Ungated sample with 35.14% of the events inside the fluorescence gate in FL-2. (F) Sample gated on R1orR2 with 3.29% of the events in the fluorescence gate in FL-2 channel. Note the reduction in background fluorescence after gating.

A similar situation is seen in two colour analysis. Fig. 5-9 shows an example of an unstained non-parenchymal sample observed in FL2 versus FL1 before and after gating on R1orR2.

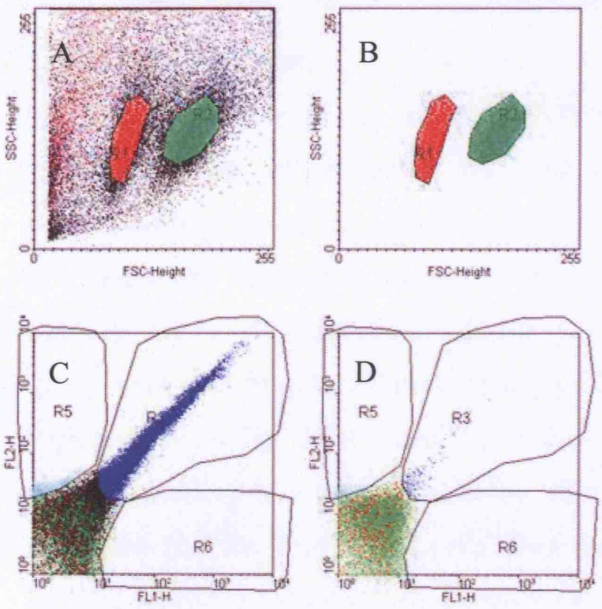


Fig. 5-9 Two-colour FCM analysis of an unstained non-parenchymal cell sample. (A) ungated SSC versus FSC dot-plot. The two populations of interest can be seen in red and green. (B) R1orR2 gated SSC versus FSC dot-plot. (C) ungated sample with 30.87% of the events inside the fluorescence gate R3 [double positive events]. (D) Sample gated on R1orR2 with 1.22% of the events inside the fluorescence gate in R3. Note the reduction in background fluorescence after gating.

The R1orR2 gates were used for all experiments except the CD117/CD133 experiments. The gates were kept a constant size but moved according to the distribution of the cells in the biological samples.

5.4.7 Functional calcein-AM assay

Acetoxymethyl ester derivatives of calcein (calcein-AM) are actively excluded by p-glycoprotein (Homolya et al., 1993). The accumulation of the fluorescent cell viability marker can be effectively used for studying P-glycoprotein function as calcein-AM is not fluorescent and free calcein is not a substrate of the multidrug transporter. The assay is readily applicable for kinetic studies of p-glycoprotein function (Hollo et al., 1994).

The cells were incubated with fumitremorgin C (FTC), an ABCG2 inhibitor, and PSC833, P-glycoprotein inhibitor, together with calcein-AM. Calcein-AM is taken up by the cell, where the intracellular esterases will cleave the AM moiety, producing fluorescent calcein. Normally however, calcein-AM is actively pumped out by cells by a multidrug resistance pump and the accumulation of the fluorescent product is slow. If the pump, however, is blocked, the calcein remains inside the cell which fluoresces in

the FL1 channel. The calcein-AM functional assay will therefore provide information on the functionality/pumping action of the protein pumps.

5.5 Results

5.5.1 ABCG2 analysis

5.5.1.1 FCM analysis with an ABCG2-specific antibody

5.5.1.1.1 Positive control

The human colon carcinoma cell line HT-29 was used as a positive control for ABCG2. However, a low percentage of the cells were staining (around 10%) which was considered insufficient for a positive control cell line [Fig. 5-10 A and B]. In order to investigate why the control was not working, a different set of HT-29 cells were thawed out [Fig. 5-10 C-F]. Un-trypsinised cells (i.e. loose cells derived from a very confluent flask) were used in order to exclude any problems that trypsin might cause to the cell surface antigen [Fig. 5-10 C and D]. It was discovered that in the second set of cells, ABCG2 staining was seen in both the un-trypsinised and the trypsinised cells. This suggested that the first set of cells used for these experiments might have lost the antigen, which might be due to a higher passage number (information unavailable) or some unknown selection pressure.

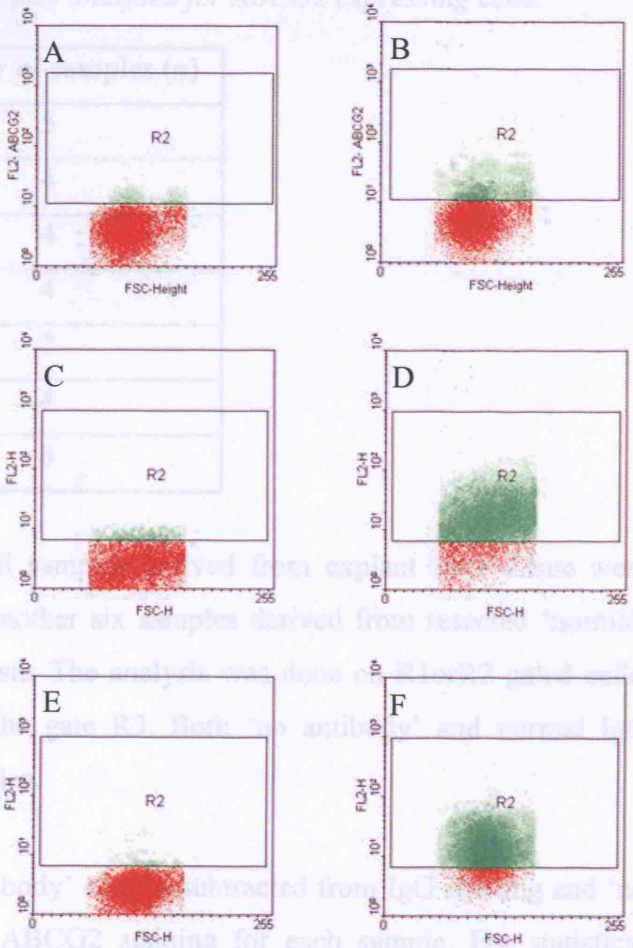


Fig. 5-10 FCM dot-plots of HT-29 cells stained with ABCG2-PE. (A) 'no antibody' control for first set of cells [R2=2.10%] and (B) cells stained with ABCG2 [R2=12.26%]. (C) 'no antibody' control of untrypsinised second set of cells [R2=1.02%] and (D) cells stained with ABCG2 [R2=63.24%]. (E) 'no antibody' control of trypsinised second set of cells [R2=0.30%] and (F) cells stained with ABCG2 [R2=65.01%].

5.5.1.1.2 Isotype matched control

Positive control HT-29 cells were stained with an isotype-matched IgG_{2B} PE-linked control antibody raised against an irrelevant antigen to assess non-specific binding. The background staining with the IgG was 6.24%.

Due to the relatively high background staining of the IgG control, a biological negative control was analysed with both ABCG2 and the control antibody. HepG2 cells were found to have very little non-specific staining 0.49% with IgG and 0.20% with the ABCG2 antibody.

Six non-parenchymal cell samples of different disease aetiologies were stained with the control antibody. The background was found to range from 4.42% to 9.87% with a mean of 6.12% (SD±2.23%). Due to the high background staining, all samples were stained both with the IgG control and the ABCG2 antibody.

5.5.1.1.3 Non-parenchymal cell samples

Table 5-2 Non-parenchymal cell samples analysed for ABCG2 expressing cells.

Disease aetiology	**Number of samples (*n*)**
ALD	5
PBC	4
PSC	4
Cryptogenic	4
Sub-fulminant	2
Fulminant	4
BS resection	6

A total of 23 non-parenchymal cell samples derived from explant liver tissue were analysed for ABCG2 expression. Another six samples derived from resected 'normal' liver tissue were used for the analysis. The analysis was done on R1orR2 gated cells. Positive events were recorded in the gate R3. Both 'no antibody' and normal IgG controls were used for all liver samples.

The results are expressed as 'no antibody' control subtracted from IgG staining and 'no antibody' control subtracted from ABCG2 staining for each sample. For statistical

analysis the disease groups were averaged and the standard deviation was calculated for all sample groups with four or more samples. Significant differences were calculated using one-way ANOVA at confidence intervals of 95% with the Tukey-Kramer post test. Fig. 5-11 shows representative dot-plots with high and low ABCG2 staining samples.

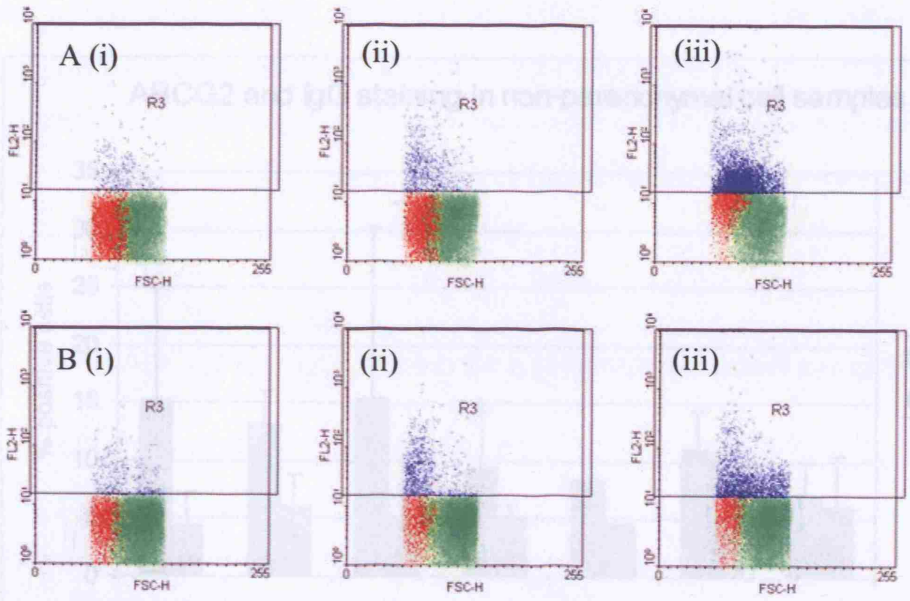


Fig. 5-11 Representative dot-plots for ABCG2 analysis.

(A) PSC and (B) sub-fulminant non-parenchymal cell samples. (i) 'no antibody' control, (ii) IgG control and (iii) ABCG2 staining. PSC with 34.36% and sub-fulminant with 6.93% of ABCG2 positive cells.

Fig. 5-12 shows a graph for the different liver samples. The percentage of ABCG2 expressing cells ranged from 3.69% to 37.19% in diseased samples and from 1.05% to 9.70% in BS samples. There was no significant difference between the groups and large standard deviations were observed. However, the mean of the BS samples was lower than the diseased samples. The IgG controls showed relatively consistent staining throughout the sample groups.

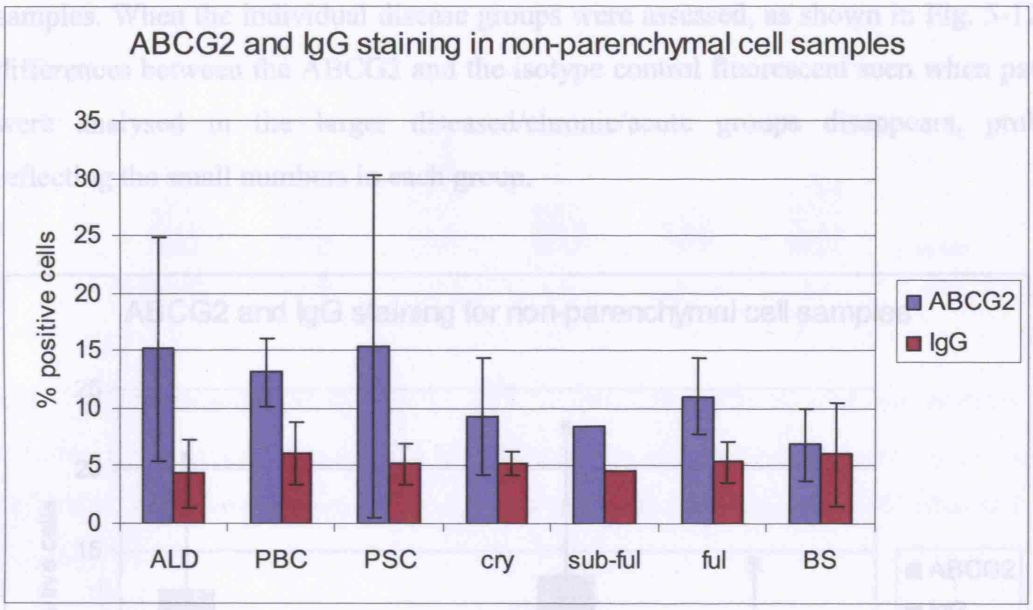


Fig. 5-12 Percentage of ABCG2 positive cells and IgG non-specific staining in non-parenchymal cell samples. Mean and standard deviation of the different sample groups are shown. No significant difference was seen between the different disease aetiologies or BS resected liver samples.

The diseased livers were grouped together and sub-divided into chronic (ALD, PBC, PSC and cryptogenic) and acute (sub-fulminant and fulminant) liver samples [Fig. 5-13]. BS livers had a lower mean compared to the other groups. Furthermore, significant differences were observed between the ABCG2 staining and IgG controls (i.e. ABCG2 *versus* IgG) within each disease group (diseased, chronic and acute) but not in the BS samples. The failure to find any differences between specific and non-specific antibody associated fluorescence in the BS group indicates that, at least with the technique used here, there are no ABCG2 positive cells detectable in the BS samples. When the individual disease groups were assessed, as shown in Fig. 5-12, the differences between the ABCG2 and the isotype control fluorescent seen when patients were analysed in the larger diseased/chronic/acute groups disappears, probably reflecting the small numbers in each group.

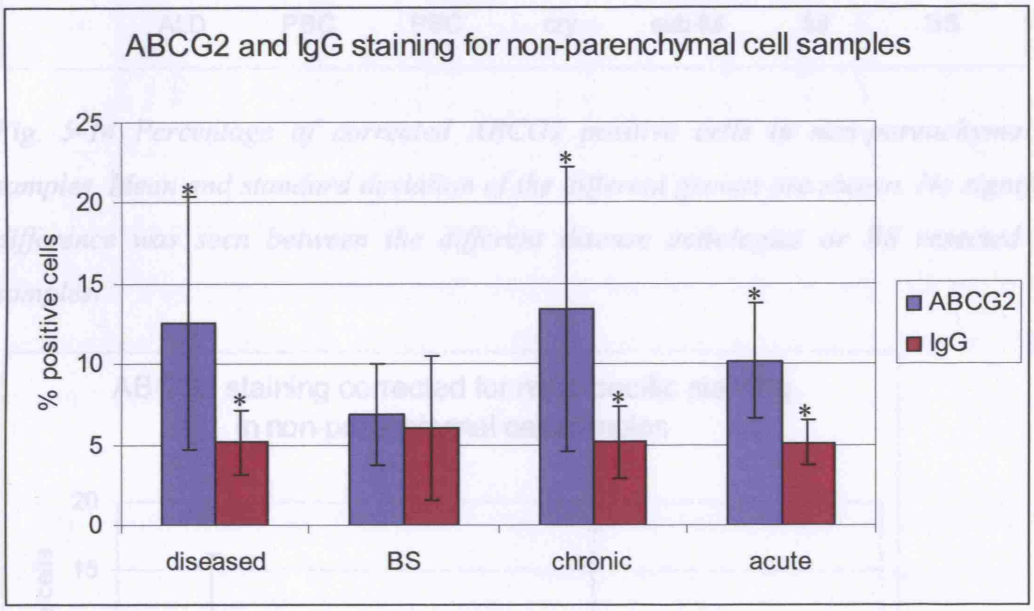


Fig. 5-13 Percentage of ABCG2 positive cells with their corresponding IgG controls in non-parenchymal cell samples divided into diseased, BS, chronic and acute liver samples. Mean and standard deviation of the different groups are shown. No significant differences are observed between the different sample groups, but significant differences were observed between the ABCG2 positive cells and the IgG controls for diseased, chronic and acute liver samples ($p<0.05$), but not for the BS samples and the IgG control.*

This data is graphed differently in Fig. 5-14 and Fig. 5-15, with the isotype data subtracted from each sample.

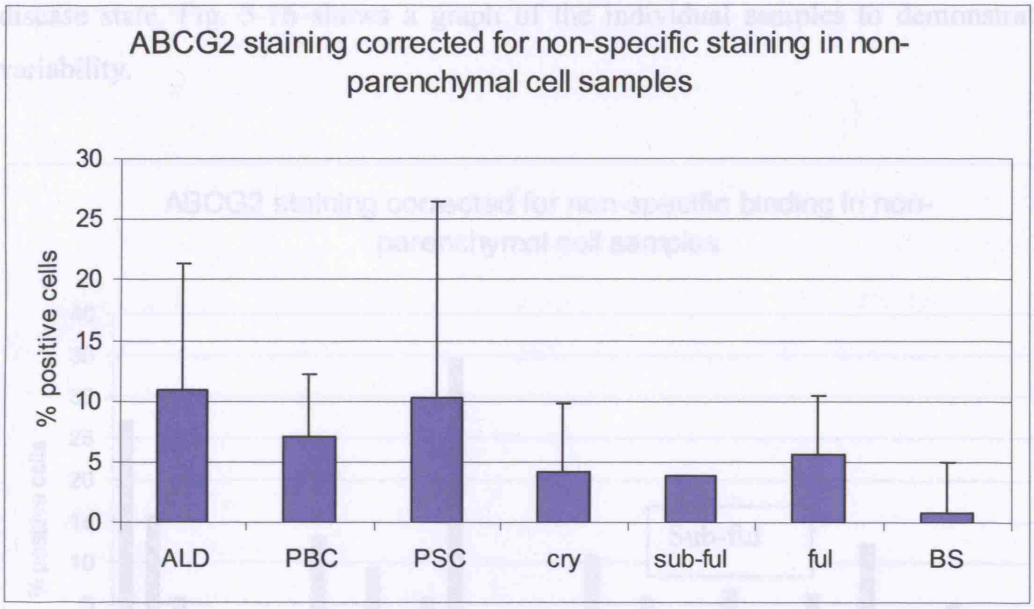


Fig. 5-14 Percentage of corrected ABCG2 positive cells in non-parenchymal cell samples. Mean and standard deviation of the different groups are shown. No significant difference was seen between the different disease aetiologies or BS resected liver samples.

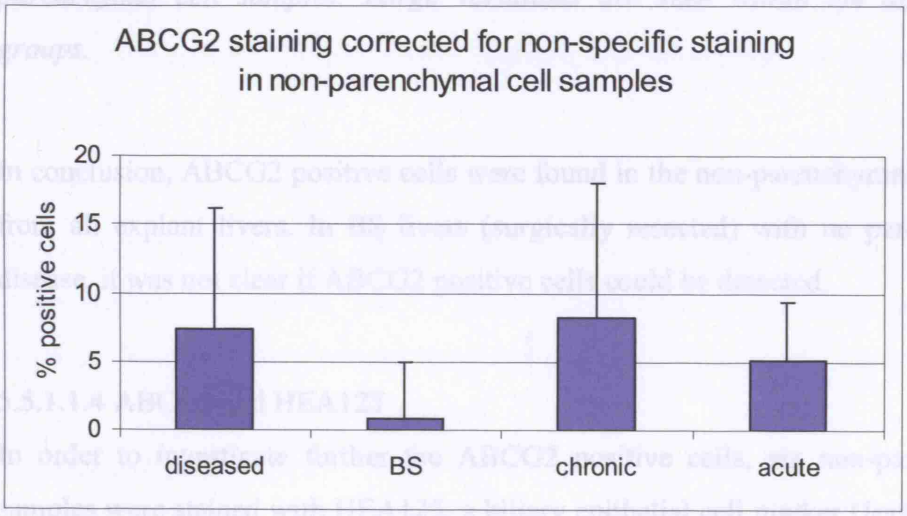


Fig. 5-15 Percentage of corrected ABCG2 positive cells in non-parenchymal cell samples divided into diseased, BS, chronic and acute liver samples. Mean and standard deviation of the different groups are shown. No significant difference was seen between the different groups.

Large standard deviations were observed due to considerable differences in the percentage of ABCG2 positive cells within the sample groups. It is possible that ABCG2-expression in the different samples were dependent on a factor other than disease state. Fig. 5-16 shows a graph of the individual samples to demonstrate the variability.

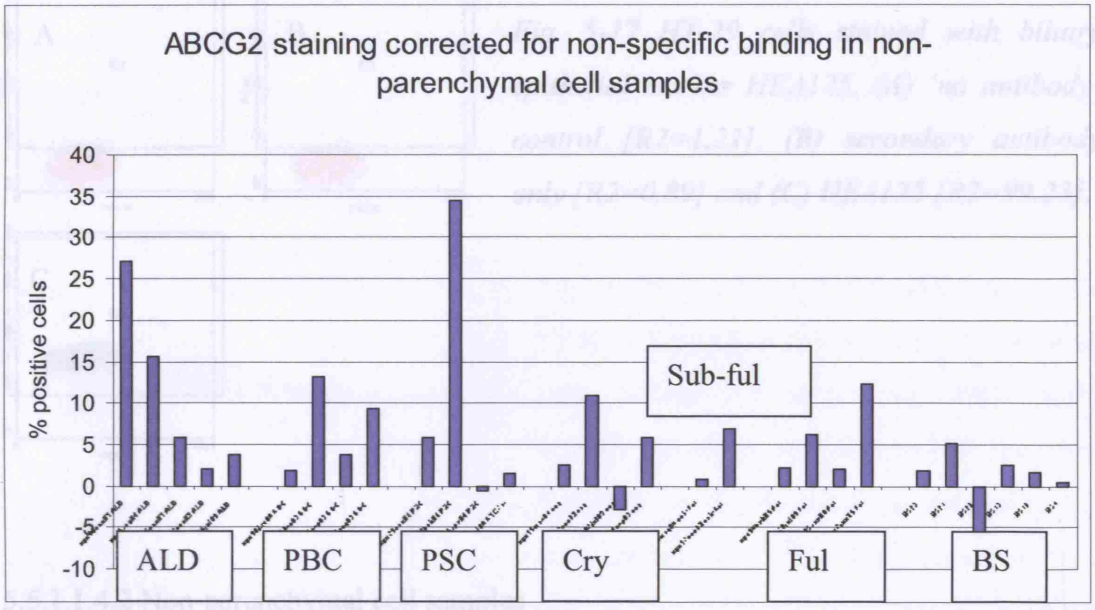


Fig. 5-16 Percentage of corrected ABCG2 positive cells in all individual non-parenchymal cell samples. Large variations are seen within the different disease groups.

In conclusion, ABCG2 positive cells were found in the non-parenchymal cells analysed from all explant livers. In BS livers (surgically resected) with no parenchymal liver disease, it was not clear if ABCG2 positive cells could be detected.

5.5.1.1.4 ABCG2 and HEA125

In order to investigate further the ABCG2 positive cells, six non-parenchymal cell samples were stained with HEA125, a biliary epithelial cell marker (Joplin et al., 1989; Joplin et al., 1990). This was analysis was performed to examine if biliary cell contamination could have led to the presence of ABCG2 positive cells.

5.5.1.1.4.1 HEA125 control

HT-29 cells were used as a positive control for the HAE-125 antibody. 98.34% of the cells stained positive and a clear shift of the cells was seen [Fig. 5-17]. No isotype-matched control was available for the antibody.

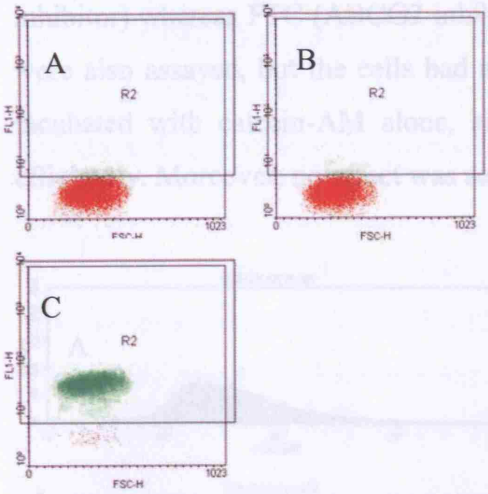


Fig. 5-17 HT-29 cells stained with biliary epithelial marker HEA125. (A) 'no antibody' control [R2=1.21], (B) secondary antibody only [R2=0.89] and (C) HEA125 [R2=99.23].

5.5.1.1.4.2 Non-parenchymal cell samples

Six non-parenchymal cell samples were analysed for the expression of HEA125 in order to compare it with ABCG2 staining. Table 5-3 shows the percentage of cells expressing the two antibodies. No correlation between the expression of the two antigens is observed.

Table 5-3 HEA125 and ABCG2 positive cells analysed on the same six non-parenchymal cell samples. ABCG2 samples have been corrected for non-specific IgG binding. No correlation between the two was seen in the assayed samples.

	HEA125	ABCG2
npc25nov03 ALD	18.61	5.8
npc5aug04 ALD	11.09	15.59
npc30jan04 PBC	2.09	1.78
npc8dec03 PBC	1.26	13.11
BS18	2.84	1.69
BS19	3	2.51

5.5.1.2 Calcein-AM functional assay for ABCG2 and P-glycoprotein

5.5.1.2.1 Positive control

The cell line CEM/VLB was used as positive control for the calcein-AM functional assay. Fig. 5-18 shows histograms of the CEM/VLB cells with and without the inhibitors. The assay showed inhibition of the pump with the PSC (P-glycoprotein inhibitor) whereas FTC (ABCG2 inhibitor) did not affect it. Cell lines A549 and HT-29 were also assayed, but the cells had a higher fluorescence than CEM/VLB cells when incubated with calcein-AM alone, suggesting the calcein-AM was not pumped out efficiently. Moreover, no effect was seen with the inhibitors.

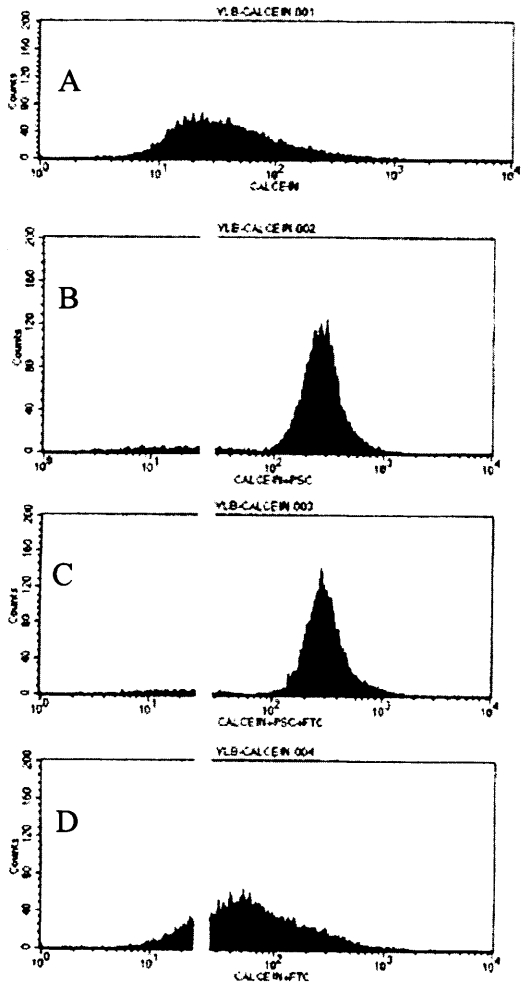


Fig. 5-18 Calcein-AM functional assay on CEM/VLB positive control cells. (A) control with calcein-AM only. The cells were actively pumping out calcein-AM and low fluorescence is observed in the cells. (B) cells incubated with PSC inhibitor and calcein-AM. The cells were unable to pump out calcein-AM and become fluorescent which was observed by a shift of the cell population to the right in the FL-1 channel. (C) cells incubated with both PSC and FTC inhibitors showed a similar trend to PSC alone. (D) cells incubated with FTC alone. FTC did not seem to have a significant inhibitory effect on the cells as they were still able to pump out most of the calcein-AM and had low fluorescence. A very slight shift to the right can be seen in the FL-1 channel.

5.5.1.2.2 Non-parenchymal cell samples

Despite the failure to find a positive control cell line that would demonstrate inhibition of the ABCG2-pump, the calcein-AM assay was tested in non-parenchymal cell samples.

Four samples were analysed and all samples showed higher starting fluorescence than with the CEM/VLB control cells, suggesting calcein-AM was not pumped out as effectively. None of the samples showed increased fluorescence with either of the two inhibitors. Fig. 5-19 shows a representative non-parenchymal sample.

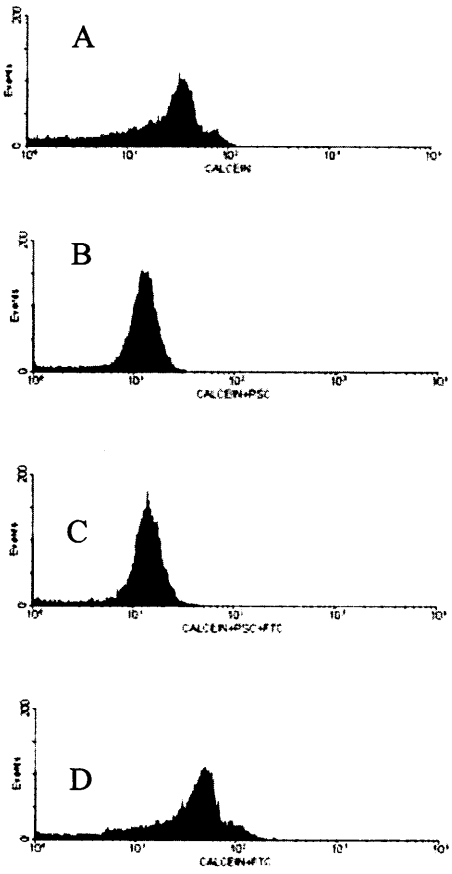


Fig. 5-19 Calcein-AM assay on a representative non-parenchymal cell sample [PBC]. (A) control with calcein-AM only. The cells were not actively pumping out calcein-AM as efficiently as CEM/VLB cells and the fluorescence of the cells was higher than with the control (acquisition settings changed in order to be able to observe fluorescence increase with inhibitors). (B) cells incubated with PSC inhibitor (C) cells incubated with both PSC and FTC inhibitors and (D) cells incubated with FTC alone. No increased fluorescence was observed with any of the inhibitors.

5.5.2 Cmet/CD49f analysis

5.5.2.1 Positive controls

Peripheral blood mononuclear cells [PBMCs], isolated from normal human blood, were used as a positive control for the CD49f antibody. CD49f is expressed on thymocytes, T lymphocytes and monocytes. Fig. 5-20 shows CD49f expression in blood cells using a FITC-labelled secondary antibody.

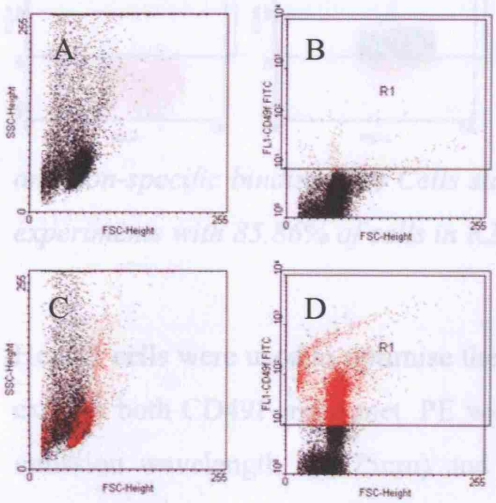


Fig. 5-20 FCM dot-plots of PBMCs. (A) SSC versus FSC for 'secondary antibody only' control showing cell populations in the blood sample. (B) FL1 versus FSC for 'secondary only' control showing background fluorescence of the cells. Cells in R1 are counted as positive events. The background consists of 0.82% of the cells in R1. (C) SSC versus FSC for the CD49f stained sample and (D) FL1 versus FSC for the CD49f stained sample. 57.70% of the cells express CD49f (inside R1) compared to the background of 0.82%. (C) provides information on where these positive cells are found on the SSC versus FSC dot-plot in red.

HepG2 cells were used as a positive control for c-met. Several antibodies were tested without success before this polyclonal antibody was found. Fig. 5-21 shows a comparison between an unsuccessful antibody DO-24 (Wormstone et al., 2000) and the antibody used in the experiments. The clone DL-21 was also tried but with similar results to clone DO-24.

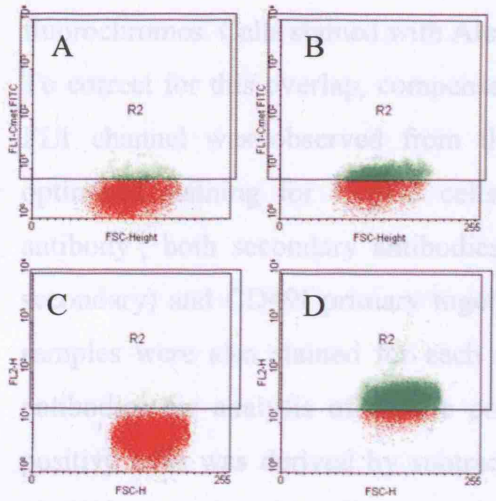


Fig. 5-21 FCM dot-plots (FL1 versus FSC) of HepG2 cells. (A) Secondary antibody only control for c-met antibody clone DO-24 with 4.28% of cells in R2 representing background fluorescence and non-specific binding. (B) cells stained with DO-24 with 42.70% of cells in R2. (C) Secondary antibody only control for polyclonal c-met antibody with 0.32% of cells in R2 representing background fluorescence and non-specific binding. (D) Cells stained with polyclonal c-met used in the following experiments with 85.86% of cells in R2.

HepG2 cells were used to optimise the acquisition for two-colour analysis. HepG2 cells express both CD49f and c-met. PE was used as a secondary antibody for CD49f (peak emission wavelength at 575nm) and AlexaFluor 488 (peak emission wavelength at 519nm) as a secondary for c-met. These two fluorochromes have overlapping emission spectra and therefore the acquisition settings required compensation. Fig. 5-22 shows emission spectra of different fluorochromes. (1) for AlexaFluor 488 and (2) for PE. The two graphs overlap around 550nm (Molecular Probes, 2005).

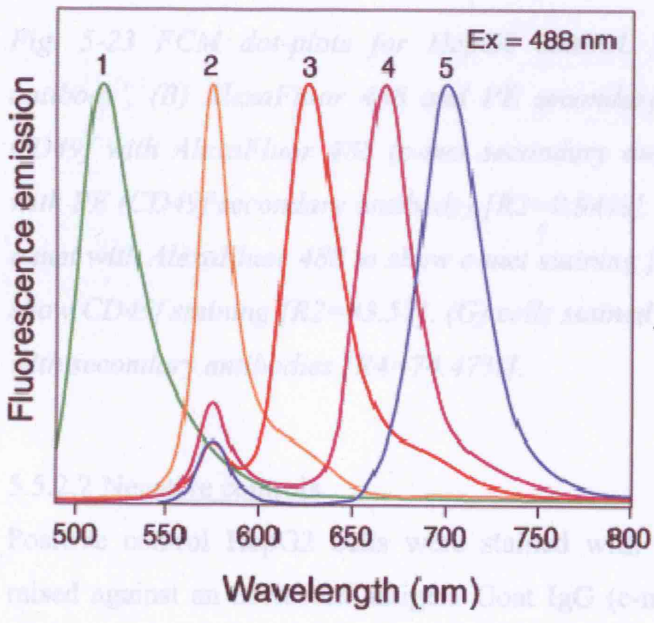


Fig. 5-22 Figure from Molecular Probes web-site (Molecular Probes, 2005) showing emission spectra of different fluorochromes. Number (1) is for AlexaFluor 488 and (2) for PE. Note the overlap of these two peaks around 550nm.

Compensation was set up in order to remove overlapping fluorescence between the two fluorochromes. Cells stained with AlexaFluor 488 showed overlap into the FL2 channel. To correct for this overlap, compensation was applied (FL2-FL1%). No overlap to the FL1 channel was observed from the cells stained with PE. Fig. 5-23 shows the optimised staining for HepG2 cells together with controls. Controls included 'no antibody', both secondary antibodies only, c-met primary together with PE (CD49f secondary) and CD49f primary together with AlexaFluor 488 (c-met secondary). The samples were also stained for each marker separately as well as together with both antibodies for analysis of double positive cells. For data-analysis the percentage of positive cells was derived by subtracting the corresponding control from the sample. 88.53% of the HepG2 cells were c-met positive, 82.63% CD49f positive and 74.36% were double positive.

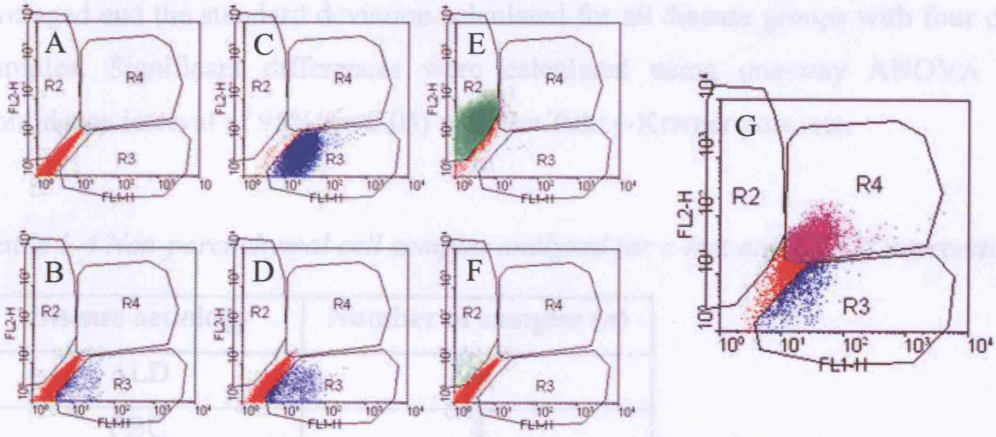


Fig. 5-23 FCM dot-plots for HepG2 control. Sample controls included (A) 'no antibody', (B) AlexaFluor 488 and PE secondary antibodies only [R4=0.11%], (D) CD49f with AlexaFluor 488 (c-met secondary antibody) [R3=9.36%] and (F) c-met with PE (CD49f secondary antibody) [R2=0.94%]. The cells were also stained with (C) c-met with AlexaFluor 488 to show c-met staining [R3=97.89%], (E) CD49f with PE to show CD49f staining [R2=83.57]. (G) cells stained with both c-met and CD49f together with secondary antibodies [R4=74.47%].

5.5.2.2 Negative controls

Positive control HepG2 cells were stained with isotype-matched control antibodies raised against an irrelevant antigen. Goat IgG (c-met control) gave 2.16% and mouse IgG_{2B} (CD49f control) 0.01% of background staining.

IgG controls were also tested on six different non-parenchymal cell samples (one from each ALD, PBC, PSC, cryptogenic, fulminant and BS) to investigate non-specific binding of the primary antibodies in the samples. The mean background produced by goat IgG (c-met control) was 0.89% (SD±1.11%) and by mouse IgG_{2B} (CD49f control) was 0.04% (SD±0.86%). These values were considered negligible for the analysis.

5.5.2.3 Non-parenchymal cell samples

Both c-met expression and CD49f expression were analysed separately on 22 non-parenchymal cell samples and six BS liver samples [Table 5-4]. Analysis was done in R1orR2 gated cells. Positive events were recorded in the gate R3. 'No antibody' and 'secondary antibody only' controls were used for all samples and the percentage of positive cells was assessed by subtracting the 'secondary antibody only' control from the stained sample. The c-met and CD49f expression in each sample group was averaged and the standard deviation calculated for all disease groups with four or more samples. Significant differences were calculated using one-way ANOVA at the confidence interval of 95% ($p<0.05$) with the Tukey-Kramer post test.

Table 5-4 Non-parenchymal cell samples analysed for c-met and CD49f expression.

Disease aetiology	**Number of samples (*n*)**
ALD	4
PBC	4
PSC	4
Cryptogenic	4
Sub-fulminant	2
Fulminant	4
BS resection	6

5.5.2.3.1 Single colour analysis for c-met

Fig. 5-24 shows a representative diseased sample and BS sample stained with c-met.

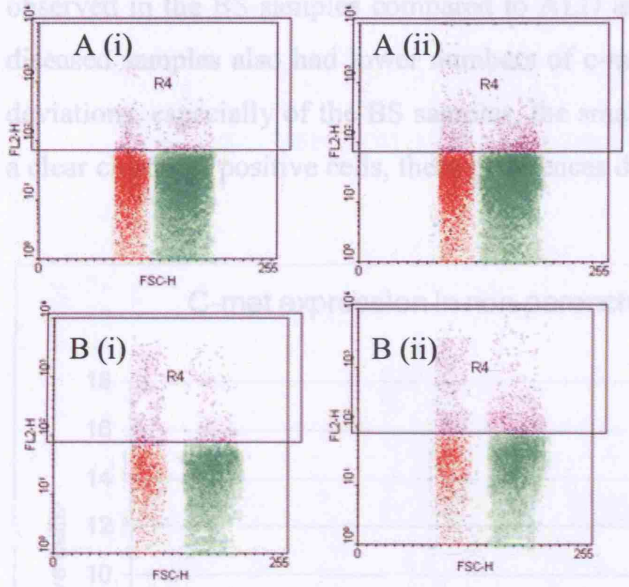


Fig. 5-24 Showing c-met expression for a representative (A) diseased [PBC] and (B) BS sample. (i) control sample and (ii) sample stained with c-met. No clear c-met positive population is seen. BS samples appear to have more background staining than diseased samples.

C-met expression in the different disease groups are shown in Fig. 5-25. The number of c-met positive cells in diseased samples range from 0.01% to 5.39% and in the resected BS samples from 2.01% to 19.29%. Significantly more c-met positive cells were observed in the BS samples compared to ALD and fulminant samples ($p<0.05$). Other diseased samples also had lower numbers of c-met cells, but due to the large standard deviations, especially of the BS samples, the small numbers of cells and the absence of a clear cluster of positive cells, these differences did not reach significance.

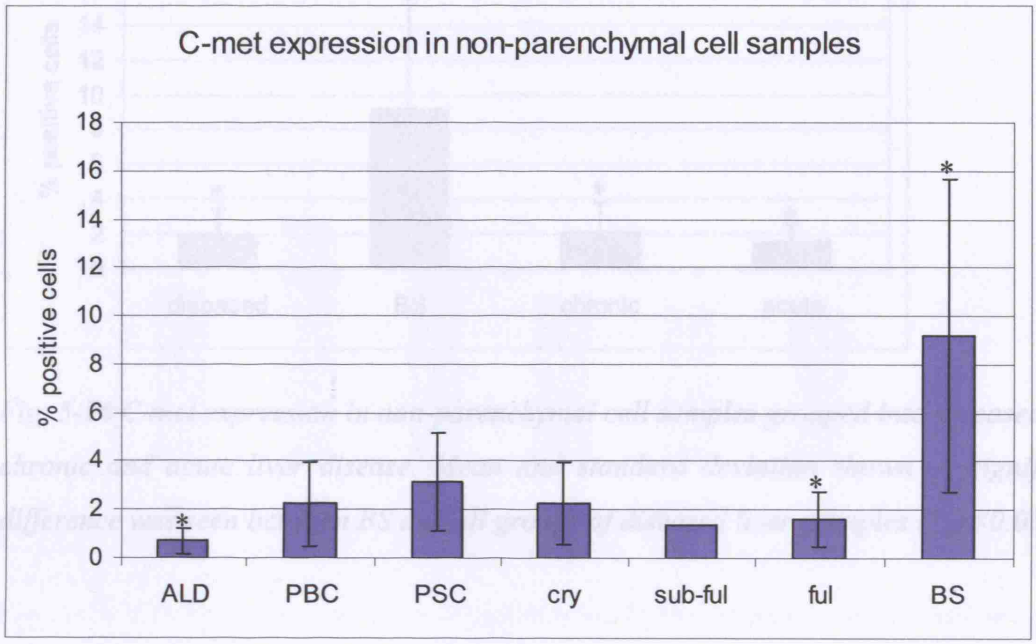


Fig. 5-25 C-met expression in non-parenchymal cell samples. Mean and standard deviation shown for each disease group. A significant difference was seen between ALD and fulminant livers compared to the BS samples ($p<0.05$).*

However, when diseased samples were grouped together and sub-divided into chronic (ALD, PBC, PSC and cryptogenic) and acute (sub-fulminant and fulminant) liver disease, significant differences were observed between BS liver resection samples and all the diseased groups [Fig. 5-26].

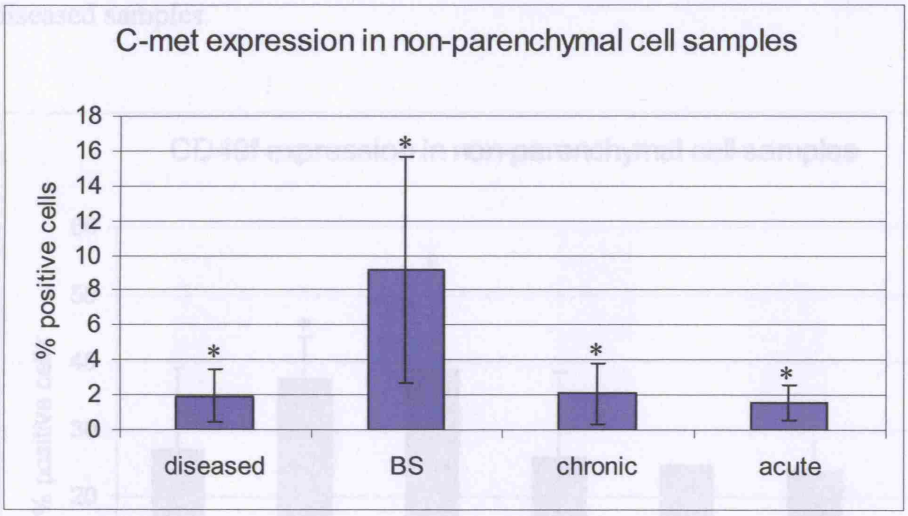


Fig. 5-26 C-met expression in non-parenchymal cell samples grouped into diseased, BS, chronic and acute liver disease. Mean and standard deviation shown. A significant difference was seen between BS and all groups of diseased liver samples ($p<0.05$).*

5.5.2.3.2 Single colour analysis for CD49f

Fig. 5-27 shows a representative diseased sample and BS sample stained with CD49f.

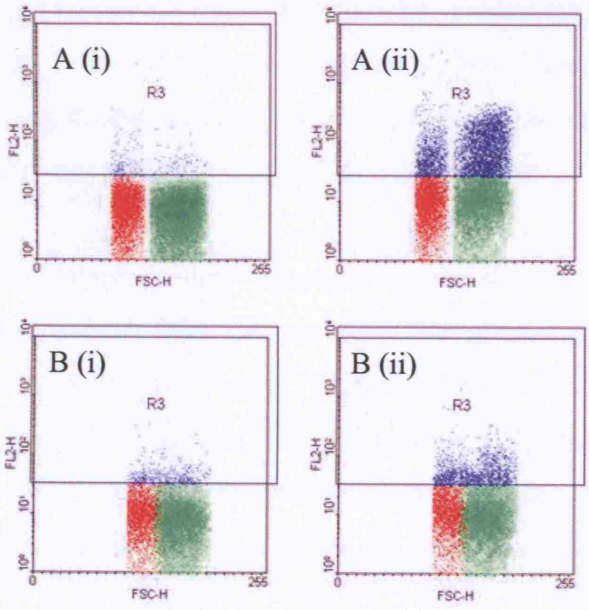


Fig. 5-27 Showing CD49f expression for a representative (A) diseased [PBC] and (B) BS sample. (i) control sample and (ii) sample stained with CD49f. A relatively distinct population of CD49f positive population was seen in the diseased samples and a relatively large proportion of cells were staining positive in both samples.

CD49f expression in the different disease groups are shown in Fig. 5-28. Significantly more CD49f cells were observed in PBC and PSC liver samples compared to BS livers ($p<0.05$). CD49f positive cells ranged from 12.05% to 53.83% in the diseased samples and 6.64% to 26.75% for the BS samples. Although no definite clusters of cells were observed, a relatively distinct and convincing population was seen especially in the diseased samples.

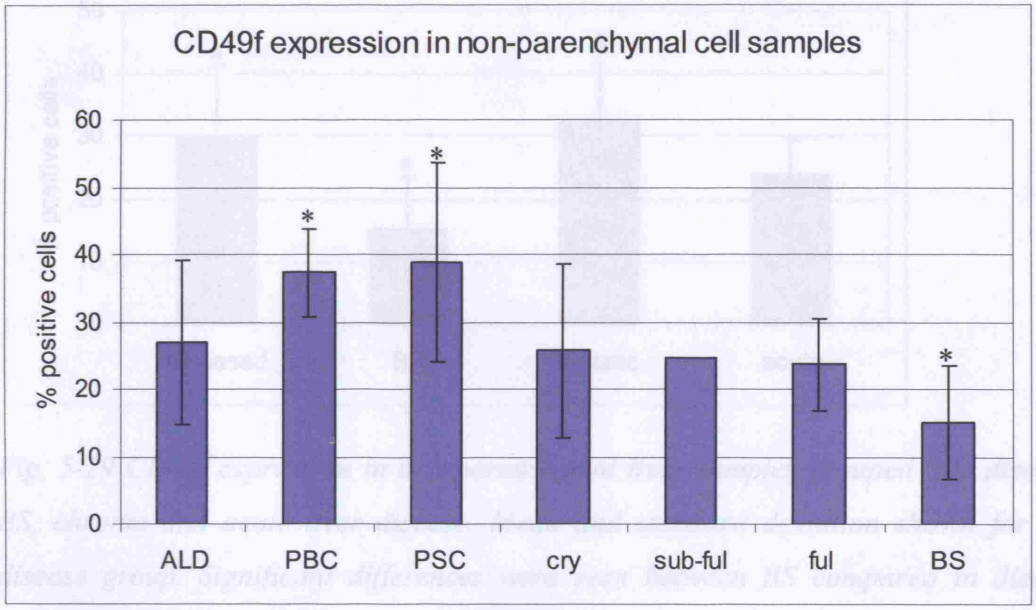


Fig. 5-28 CD49f expression in non-parenchymal liver samples. Mean and standard deviation shown for each disease group. Significant differences were seen between PBC and PSC samples compared to BS ($p<0.05$).*

Fig. 5-29 shows samples grouped into diseased, BS, chronic and acute liver disease. Interestingly, significant differences were observed between BS liver resection samples and diseased samples and between BS and chronic liver samples. However, BS *versus* acute liver samples was not found to be significantly different.

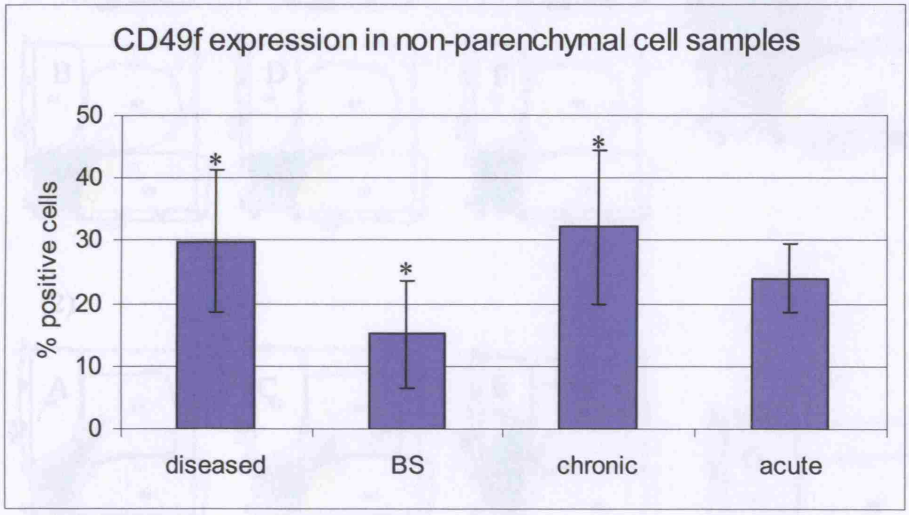


Fig. 5-29 CD49f expression in non-parenchymal liver samples grouped into diseased, BS, chronic and acute liver disease. Mean and standard deviation shown for each disease group. Significant differences were seen between BS compared to diseased samples and chronic ($p<0.05$).*

5.5.2.3.3 Double c-met/CD49f positive population

Eight non-parenchymal cell samples isolated from explants were analysed together with three BS samples for cells expressing both c-met and CD49f. Due to low expression of c-met, double staining was thought to aid the identification of double positive cells. If the c-met positive cells were to express CD49f, a clearer cell cluster might be observed.

The samples were acquired using the HepG2 compensation settings. Further compensation with the non-parenchymal cells was not possible due to the low number of c-met positive events. The double positive cells were also analysed with similar gates to the HepG2 control. Fig. 5-30 shows a representative explant sample and BS sample for the two-colour analysis. A strongly staining artefact was observed in all BS samples when stained with AlexaFluor 488 (seen in gate R7).

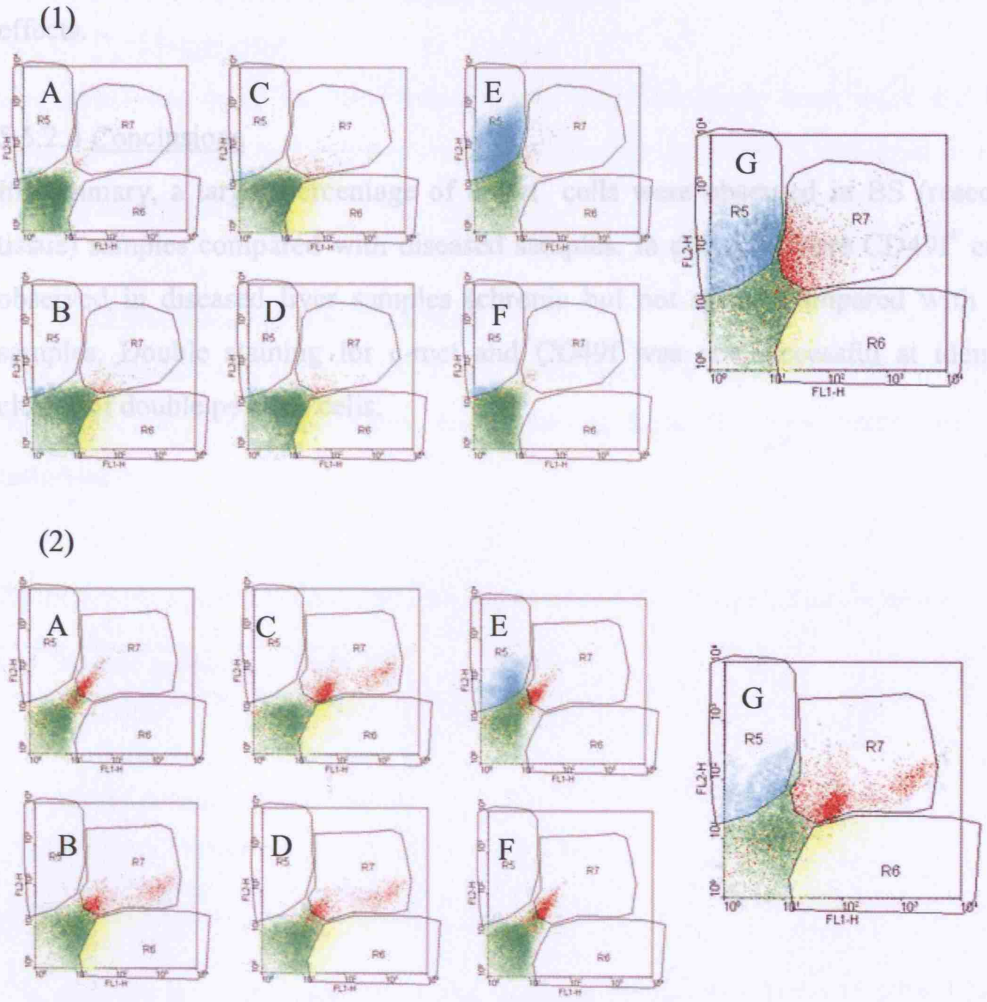


Fig. 5-30 FCM dot-plots for (1) a representative explant sample and (2) a representative BS sample. Sample controls included (A) 'no antibody', (B) AlexaFluor 488 and PE secondary antibodies only (D) CD49f with AlexaFluor 488 (c-met secondary antibody) and (F) c-met with PE (CD49f secondary antibody). The cells were stained with (C) c-met with AlexaFluor 488 to show c-met staining and (E) CD49f with PE to show CD49f staining alone. (G) cells stained with both c-met and CD49f together with secondary antibodies. Artefact population of cells seen in R7 in BS samples. The population is seen in all BS samples stained with AlexaFluor 488.

When Fig. 5-30 is carefully analysed, it can be seen that the background effect of the AlexaFluor 488 (D) causes a shift of the cells into the double positive gate when it is combined with the real staining of CD49f (E). Therefore no real double-positive population is seen. In fact, more double positive cells are observed than single c-met^{+} cells. It was assumed that the experimental set-up was not sensitive enough to analyse

double positive events (if they did exist) due to compensation and other background effects.

5.5.2.4 Conclusions

In summary, a larger percentage of c-met$^+$ cells were observed in BS (resected liver tissue) samples compared with diseased samples. In contrast, more CD49f$^+$ cells were observed in diseased liver samples (chronic but not acute) compared with BS liver samples. Double staining for c-met and CD49f was not successful at identifying a cluster of double positive cells.

5.5.3 In search of the $CD117^{+}CD133^{+}$ population

5.5.3.1 Positive control

The antibodies used in these experiments had previously been used by Dr Mark Lowdell. A representative non-parenchymal liver sample (PBC) was used to determine the acquisition parameters.

5.5.3.2 Non-parenchymal liver samples

Five liver samples with different disease aetiologies were chosen for the analysis, listed in Table 5-5. Non-parenchymal cells isolated from BS liver resections were not analysed.

Table 5-5 Non-parenchymal cell samples used in CD117/CD133 experiments.

Disease aetiology	GCSF
PBC	No
ALD	No
Sub-fulminant	No
Giant cell hepatitis	Yes
Cryptogenic	No

The samples were analysed by cluster analysis on the FL3 (CD117-PC5) versus FL2 (CD133-PE) channel. Double positive events, as well as, $CD117^{+}CD133^{neg}$ and $CD117^{neg}CD133^{+}$ events were evaluated. Fig. 5-31 shows FCM dot-plots of the samples analysed. The gates were redrawn for each sample. A 'no antibody' control and a CD45 stained negative control was used for the PBC explant. The CD45 control was needed in order to get the same acquisition parameters as the samples, i.e. $CD45^{low}$ cells. Consequently the 'no antibody' control is not directly comparable to the stained samples. Only the PBC sample was used for the controls, to give an idea of background events.

$CD117^{+}CD133^{neg}$ cells are seen as a cluster in all the samples analysed. A much smaller $CD117^{high}$ population is observed in addition to the $CD117^{low}$ population. A much smaller cluster is seen for the $CD133^{+}CD117^{neg}$ cell populations in all the samples. No such populations exist in the control samples. It is difficult to determine $CD117^{+}CD133^{+}$ cells, due to the autofluorescence which is also observed in the control samples.

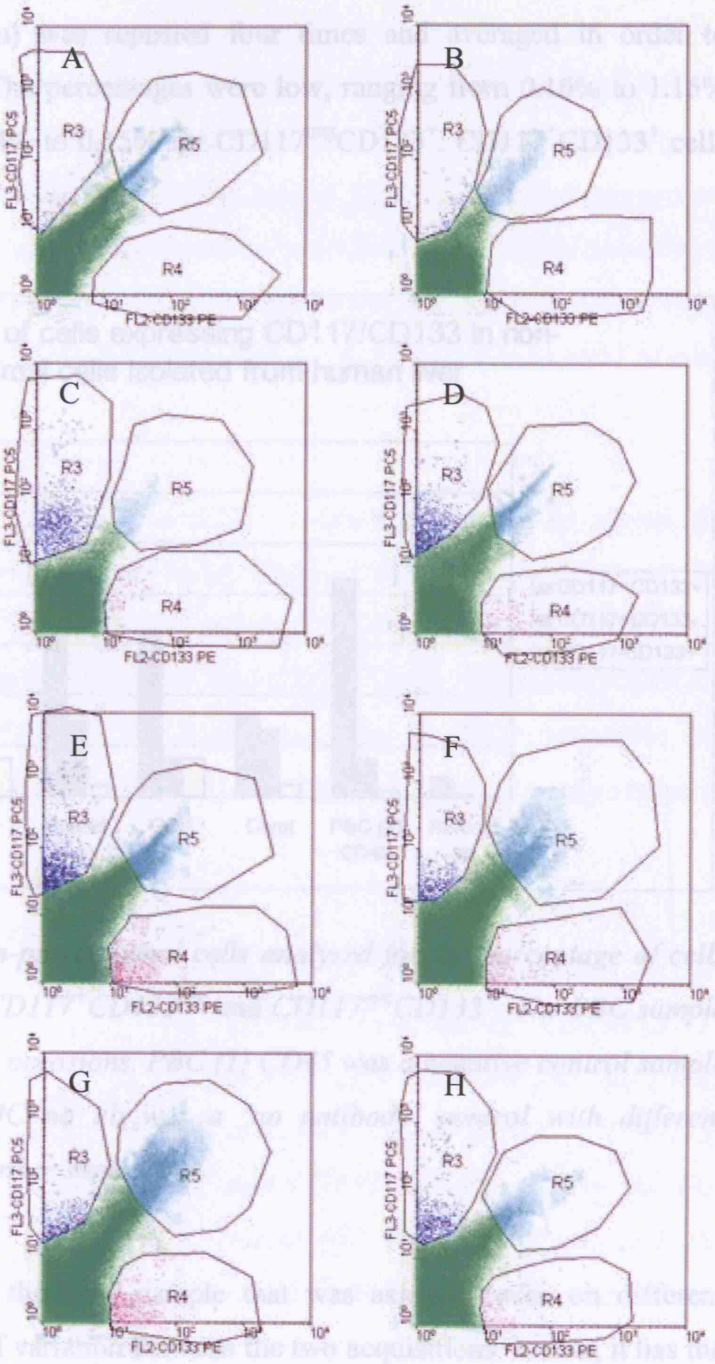


Fig. 5-31 FCM dot-plots for non-parenchymal cell samples analysed for CD117 and CD133 expression. Cells gated in R3 are $CD117^{+}CD133^{neg}$ and cells in R4 are $CD117^{neg}CD133^{+}$. R5 gates $CD117^{+}CD133^{+}$ cells. (A) 'no antibody' control for PBC liver sample. (B) CD45 negative control for PBC liver sample. (C) PBC sample acquisition 1. (D) PBC acquisition 2. (E) ALD sample. (F) sub-fulminant sample. (G) giant cell hepatitis sample. (H) cryptogenic liver sample.

The percentage of the different cell populations for each liver is shown in Fig. 5-32. Analysis (but not acquisition) was repeated four times and averaged in order to minimise error from gating. The percentages were low, ranging from 0.16% to 1.15% for $CD117^{+}CD133^{neg}$ and 0.04% to 0.15% for $CD117^{neg}CD133^{+}$. $CD117^{+}CD133^{+}$ cells ranged from 0.27% to 1.23%.

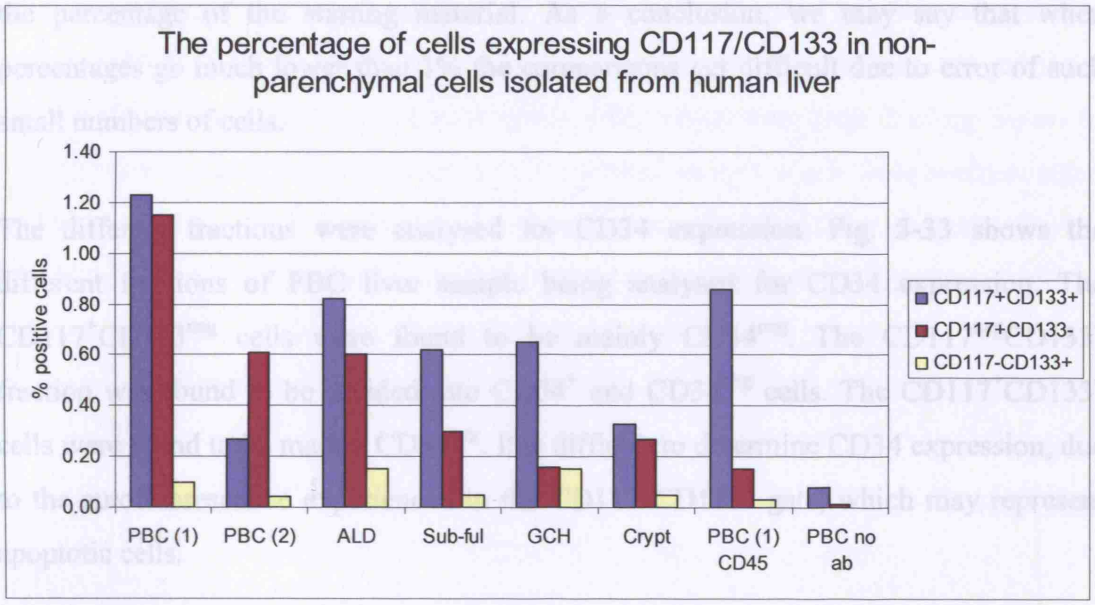


Fig. 5-32 Five samples of non-parenchymal cells analysed for the percentage of cells expressing $CD117^{+}CD133^{+}$, $CD117^{+}CD133^{neg}$ and $CD117^{neg}CD133^{+}$. The PBC sample was assayed twice on different occasions. PBC (1) CD45 was a negative control sample stained only with CD45. PBC no ab was a 'no antibody' control with different acquisition parameters to the other samples.

It is interesting to note that the PBC sample that was assayed twice on different occasions has large amounts of variation between the two acquisitions. In fact, it has the highest and lowest percentage of double positive cells. This variation between two experiments is a problem when analysing highly heterogeneous samples and when dealing with very rare events under complex analysing parameters. It is difficult to draw any firm conclusions from the double positive cells due to the high background and this variability in one sample. The two single positive populations ($CD117^{+}CD133^{neg}$ and $CD117^{neg}CD133^{+}$) form clusters that are more easily analysed and have lower values in the PBC CD45 control. Variability between the two PBC acquisitions is however also seen in these populations. It seems to be difficult to investigate such small percentages

of cells. It is also important to note that these values are not the percentage of cells from the total events, but the percentage of positive cells inside both the 'cells of interest gate' and the $CD45^{low}$ gate. Cells in 'cells of interest gate' were around 55% of the starting material and the $CD45^{low}$ gate was around 23% of the 'cells of interest gate'. Therefore the cells actually acquired consisted of around 12.65% of total events present in the sample. This means that all figures should be multiplied by 0.1265 in order to get the percentage of the starting material. As a conclusion, we may say that when percentages go much lower than 1% the comparisons get difficult due to error of such small numbers of cells.

The different fractions were analysed for CD34 expression. Fig. 5-33 shows the different fractions of PBC liver sample being analysed for CD34 expression. The $CD117^{+}CD133^{neg}$ cells were found to be mainly $CD34^{neg}$. The $CD117^{neg}CD133^{+}$ fraction was found to be divided into $CD34^{+}$ and $CD34^{neg}$ cells. The $CD117^{+}CD133^{+}$ cells were found to be mainly $CD34^{neg}$. It is difficult to determine CD34 expression, due to the autofluorescence experienced in the $CD117^{+}CD133^{+}$ gate, which may represent apoptotic cells.

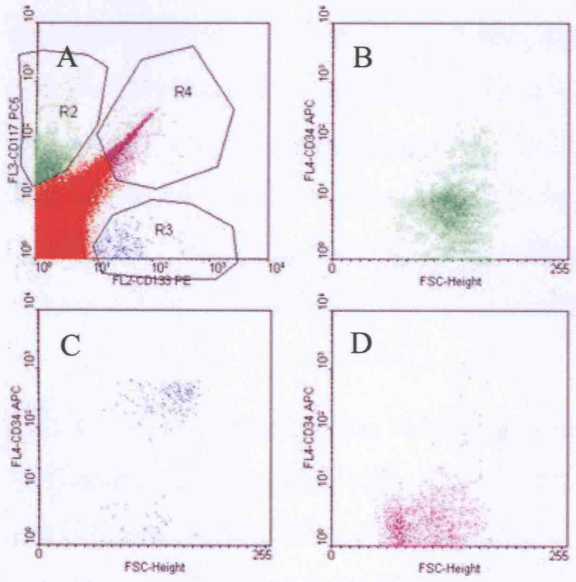


Fig. 5-33 CD34 expression of the different fractions of CD133/CD117 cells in a PBC liver sample. (A) gating of $CD117^{+}CD133^{neg}$, $CD117^{neg}CD133^{+}$ and $CD117^{+}CD133^{+}$ cells in the FL3 versus FL2 dot-plot. (B) only showing $CD117^{+}CD133^{neg}$ cells for the expression of CD34 on the y-axis. (C) only showing $CD117^{neg}CD133^{+}$ cells for the expression of CD34 and (D) only showing $CD117^{+}CD133^{+}$ cells for the expression of CD34. Only $CD117^{neg}CD133^{+}$ cells show a convincing expression of $CD34^{+}$ cells. The other two fractions may also contain some $CD34^{+}$ cells.

5.5.3.3 Conclusions and ideal/future experiments

As a conclusion, clusters of positive cells were observed, but there are variations when samples are repeated because of the very small numbers of cells analysed. Especially the dual positive events are difficult to analyse due to their location (in relation to auto-fluorescence) and how the analysis was carried out.

These experiments require a large number of cells to be analysed in order to be able to acquire 250 000 gated events. In practice this involves large volumes of labelling antibody as well as a lot of non-parenchymal cells, which were both limiting factors in our experiments. In an ideal situation a CD45 stained control would have been provided for each sample, which could then have been subtracted from the stained samples. Moreover, several repeats of the analysis would have provided information about the variation between acquisitions of one sample. More biological repeats, i.e. more livers of the same disease aetiology, would have also allowed for comparisons between different disease groups.

5.6 Discussion

Diseased and resected BS liver samples were analysed by flow cytometry for different surface markers associated with stem cells and liver progenitors. Both technical problems and cell marker expression by non-parenchymal cells are discussed.

5.6.1 FCM analysis of non-parenchymal cell samples

Flow cytometry is ideal for samples that are easily kept in a single-cell suspension, have distinct marker profiles and high expression of these markers in distinct populations. Whilst analysing non-parenchymal samples several different problems arose. Firstly, sample preparation had to be optimised to minimise clumping of the cells. Acquisition gates had to be set in a simple manner to leave maximum flexibility for the analysis. High background fluorescence was also observed in the liver samples, but this was reduced significantly (but not completely) by gating for tight populations in the SSC *versus* FSC dotplots. Any remaining background was always subtracted from the stained sample. However, in more than one colour FCM, background fluorescence was still complicating the issues of compensation and double labelled cells.

A further problem in the analysis of the percentage of positive cells was noted by the lack of distinct cell populations expressing high levels of the marker. With the markers used in this chapter, the expression was either very low (possibly non-existant) or a clear cluster of cells was not apparent (most cells expressing a marker but at different levels). This made the analysis both difficult and less reliable than if for example haematopoietic samples would have been analysed.

5.6.2 Characterisation of the starting population with stem cell markers

The non-parenchymal cells were analysed for three sets of stem cell markers. Firstly, an antibody against ABCG2 was used instead of Hoechst efflux studies, due to the damage that Hoechst 33342 has been found to cause in haematopoietic cells (Machalinski et al., 1998). ABCG2 is expressed in injured liver by oval cells in rat (Shimano et al., 2003) and ABC transporters are upregulated during human liver disease in reactive ductules (Ros et al., 2003a).

Our experiments showed the presence of ABCG2 expressing cells in the non-parenchymal cell samples of diseased but not resected liver samples (no significant

difference between BS samples stained with ABCG2 and IgG was observed). There was a significant amount of variation in the percentage of cells expressing ABCG2 between samples and no trend between the disease groups was seen. In order to investigate the possibility of the ABCG2 expressing cells being biliary epithelial cells, HEA125 was used to look at the number of biliary epithelial cells is six non-parenchymal samples already analysed for ABCG2 expression. No correlation was seen between the numbers of staining cells, with some samples having more ABCG2 positive cells than HEA125 positive cells, suggesting the ABCG2 positive cells were not merely biliary epithelial cells. Future experiments could involve double-staining non-parenchymal samples with the two antibodies to observe the number of double labelled cells. However, for enrichment of putative stem cells/progenitors, ABCG2 could be considered a marker. Even though it is unlikely that all ABCG2 positive cells would be progenitor cells due to the protective function ABC transporters provide against toxic bile constituents (Alison, 2003), an enrichment of possible progenitors may be expected.

The functionality of the ABCG2 and P-glycoprotein was assayed using the calcein-AM assay. Although the assay worked for the CEM/VLB cell line expressing P-glycoprotein, ABCG2 expressing cell lines were unable to show any fluorescent increase with inhibitors and moreover they exhibited a higher starting fluorescence than CEM/VLB cells. This is not surprising as Litman *et al.*, in fact, found that calcein-AM (or calcein) was not excluded from cell lines expressing ABCG2 (Litman et al., 2000). The calcein-AM assay in non-parenchymal cells did not show any functional P-glycoprotein.

The second antibody profile analysed on the non-parenchymal cells was c-met and CD49f expression, shown to be identified as liver progenitors in foetal mouse liver (Suzuki et al., 2002). Our experiments found the percentage of cells expressing c-met to be low and the marker was expressed at low levels. No clear population of c-met^{+} cells were observed in the diseased liver samples and it was unclear whether the low percentage of cells that were positive were due to non-specific binding, as the isotype-matched control had similar numbers of cells determined to be positive. Some BS samples had larger populations of c-met positive cells (hence the large standard deviation) and BS liver samples had significantly more positive cells compared to diseased samples. The original assumption would have been to observe more c-met positive cells in diseased liver, but it is possible c-met could be a more mature marker

and therefore not present in the starting population. Moreover, due to the nature of the BS samples, which are derived from tissue removed due to colorectal cancer, it should be noted that the c-met proto-oncogene is also associated with cancerous cells in hepatocellular carcinoma and other cancers (Ueki et al., 1997). It is therefore difficult to assess specific significance to these results. The results for c-met/CD49f double positive cells remained inconclusive in our studies. Overall, however, if the hypothesis that diseased liver is enriched in progenitor cells is correct, such progenitor cells were not found to be c-met$^+$ in our studies using flow cytometry.

On the other hand, significantly more CD49f positive cells were observed in diseased (chronic) liver samples compared with BS liver samples. Of the different liver disease aetiologies PBC and PSC reached significance. The other liver disease aetiologies showed also higher percentages of CD49f$^+$ cells (ALD>cryptogenic>sub-fulminant>fulminant) but they did not reach significance. Integrin upregulation has been observed in human liver disease (Nejjari et al., 2001) and has been found to be important both in development and differentiation of different liver cells (Couvelard et al., 1998). Although CD49f expression might be used to enrich for possible liver progenitors, the large percentages of cells expressing the marker and the lack of a clear cluster of cells (although much more apparent than with c-met positive cells), suggests it is not an ideal sorting antibody. From our data it seems that a large amount of cells express the integrin at some level and discreet positive and negative cells are difficult to establish. However, CD49f might be used as an additional marker for a putative stem cell together with other markers.

Lastly, the presence of CD117/CD133 double positive cells, identified in diseased human liver sections (Craig et al., 2004b), was analysed. These cells were known to be very rare, so a different acquisition template was set up, using CD45 expression to gate for acquisition. Although relatively clear clusters of cells were observed, the actual percentages of these cell clusters were extremely small (mainly < 1%) and variations were observed when samples were repeated. Moreover, autofluorescence affected the clear identification of CD117$^+$CD133$^+$ cells. It was therefore very difficult to compare the samples with different disease aetiologies. Moreover, these experiments required large amounts of antibodies due to the high number of cells analysed, and ideal repeats and controls were not practically achievable. Other complications arose from the autofluorescence of the starting material and the compensations involved in four-colour

analysis. Again, although it was difficult to draw any direct conclusions on the numbers of CD117 and CD133 positive cells, cell populations were clearly present and therefore cell sorting could be utilised for future experiments.

5.6.3 Conclusion and future experiments

The flow cytometric analysis of the isolated non-parenchymal cells was carried out to identify useful cell surface markers that could be utilised for cell sorting experiments. Three sets of markers (ABCG2, c-met/CD49f and CD117/CD133) were analysed in liver samples of different disease aetiologies. ABCG2, CD117 and CD133 expressing cells were identified in the non-parenchymal cell fraction. Putative c-met^{+} cells were also observed. Diseased chronic liver samples were also found to have higher percentages of CD49f^{+} cells compared to normal livers. No direct data was found to suggest that one liver disease aetiology would be more enriched in putative liver progenitors/stem cells than another (identified by these stem cell markers) but enough information was gathered for cell sorting experiments. The next chapter will investigate cell sorting possibilities using stem cell markers.

Chapter 6

Cell sorting to enrich for putative stem cells

6.1 Introduction

This chapter examines the possibilities of using cell surface markers to sort cell populations that would be enriched for putative liver stem cells or liver progenitors. Both flow cytometric cell sorting (FCMCS) and magnetic cell sorting (MACS) are investigated.

6.1.1 Cell sorting possibilities – FCMCS and MACS

The two most common cell sorting methods are FCMCS and MACS. FCMCS is based on FCM analysis, where the specific cell population to be sorted is determined with different gates. MACS, on the other hand relies on an antibody linked to a super-paramegnetic microbead. The microbead is used to pull out the cells expressing the specific antigen with a magnet. The two methods have different advantages and disadvantages.

With available equipment, FCMCS can be used with up to four different antibodies to different antigens. Cell size, granularity as well as the expression level (negative, low and high) of the selected antigens can be used to determine the cell population of interest. Moreover, two carefully defined cell populations can be selected for in one sort.

In MACS only one antigen can be used to sort at any one time. Only a 'positive' and a 'negative' cell population can be selected for and there may be a range of expression in the positive sample. There are however, possibilities of using consecutive sorts to get a more defined cell population. Depletion of a particular marker is possible as well as the use of a cocktail of different antibodies, for example the depletion of cells expressing lineage markers in a haematopoietic sample. Multi-sort kits enable subsequent positive selections by removal of the microbeads used in the first sort and the cells can then be re-stained with a different antibody for a second sort.

The advantages of MACS are mainly methodological. A simple stand with a magnet and a column are required for the sort, which can easily be performed in a standard laboratory. In contrast, FCMCS requires a very expensive instrument and an exceptionally experienced person to run it.

FCMCS, MACS and other sorting methods have been compared for purity, recovery, yield and enrichment of $CD34^+$ cells isolated from bone marrow, umbilical cord and peripheral blood (de Wynter et al., 1995). It was found that FCMCS and MACS were the most reliable methods tested with consistent high purities of >70%. MACS was found to be superior (even to FCMCS) for enrichment of colony-forming-cells (de Wynter et al., 1995). However, it is important to remember that this method only required the selection of cells expressing one marker, CD34.

The sorting method of choice mainly depends on the cell population to be sorted. For straightforward positive and negative fractions MACS is simpler, reliable and a high purity can be achieved with extra columns. However, if the marker of choice is expressed at different levels by most cells, it is clear that that the cells cannot be selected with MACS. On the other hand, due to operator drawn gates, populations with different expression levels can be isolated with FCMCS. For a combination of markers, FCMCS is also a better choice, especially if one of the markers is differentially expressed. Conversely, cell loss and the time used for the sort can be greater in FCMCS due to the methodology (and complexity) of the sort. Often MACS is used to pre-sort cells before they are sorted by FCMCS.

This introduction will briefly summarise experiments involving cell markers discussed in *Chapter 5* and their use in cell sorting, as well as, the use of methylcellulose culture assays for the analysis of progenitors.

6.1.2 Cell sorting markers

MACS sorting has successfully been used to select for $CD133^+$ cells in haematopoietic stem cell experiments. In a large scale isolation of $CD133^+$ cells from GCSF mobilised human peripheral blood the percentage of $CD133^+$ cells was enriched from 0.39-2.03% $CD133^+$ cells before selection to 85.2-98.0% after MACS sorting. The recovery was 44-100% (Gordon et al., 2003). $CD133^+$ cells have also been isolated from human foetal

brain tissue with MACS. The purity of the sorted fraction was around 85% (Yu et al., 2004).

CD117 selection has been used to isolate putative liver stem cells from rodent and human liver. From a rat model with injured liver (2-AAF/PH) $CD117^+$ oval cells were isolated with MACS. The cells formed colonies expressing albumin, CK19 or both when cultured in vitro (Qin et al., 2004).

As discussed earlier, Crosby *et al.* used CD117 and CD34 MACS to pull out putative liver stem cells from normal and diseased (ALD and PBC) human liver (Crosby et al., 2001). After 7 days culture in biliary epithelial cell culture media, the cells differentiated into biliary epithelial cells expressing CK19 and CD31 (Crosby et al., 2001).

C-met^+ cells have also been MACS sorted (Wang et al., 2005). $\beta_2$microglobulin^{neg}c-met^+ cells were isolated from human umbilical cord blood using MACS depletion and positive selection. The cells were about 2.5% of the total nucleated cells and were $CD34^{+/neg}CD90^{+/neg}CD49f^+CD29^+alb^+AFP^+$. These cells were shown to differentiate to hepatocyte-like cells expressing AFP, albumin, CYPB1B1 and CK18 and CK19 and with liver specific functions of indocyanine green uptake, ammonia metabolism and albumin secretion, by being grown on a transgenic feeder cell line expressing HGF (Wang et al., 2005).

MACS and FCMCS have been used in conjunction to isolate $CD34^+CD133^+$ cells from human cord blood, bone marrow and peripheral blood (de Wynter et al., 1998). $CD34^+$ cells were firstly isolated by MACS and the cells were then labelled with a CD133 antibody and FCMCS sorted into $CD34^+CD133^+$ and $CD34^+CD133^{neg}$ fractions (de Wynter et al., 1998).

Moreover, a very rare $Lin^{neg}CD34^+CD38^{neg}CD133^+CD7^{neg}$ cell population (0.2% of $Lin^{neg}CD34^+CD38^{neg}$) was identified in human bone marrow using lineage negative selection (Lin^{neg}) with magnetic cell sorting (containing a cocktail of lineage antibodies) followed by FCMCS (Gallacher et al., 2000).

The FCMCS isolation of the c-met^{+}CD49f$^{+/low}$CD117^{neg}CD45^{neg}TER119^{neg} cell population by Suzuki *et al.* from foetal mouse liver has been discussed in earlier chapters (Suzuki et al., 2002; Suzuki et al., 2000a). The foetal cells were analysed by fractionating a continuous CD117^{neg}CD45^{neg}TER119^{neg} cell population into CD49f^{neg}, CD49f$^{+/low}$ or CD49f$^{+/high}$ and these fractions were further divided into c-met^{+} or c-met^{neg}. The cells with highest colony forming potential, were found to be c-met^{+}CD49f$^{+/low}$ and the cells were also transplanted into recipient animals where they differentiated into hepatocytes and biliary epithelial cells (Suzuki et al., 2002).

Furthermore, c-met^{+}CD45^{neg} cells isolated from foetal rat liver by FCMCS has been shown to have repopulation capacity in damaged livers in adult rat models (Suzuki et al., 2004).

6.1.3 Methylcellulose culture

Methylcellulose is a polymer that forms a semi-solid matrix gel with good optical clarity. Methylcellulose media is used to identify different haematopoietic progenitors or colony-forming cells (CFCs) of erythrocyte, granulocyte, monocyte-macrophage cell lineages. In semi-solid media the CFCs proliferate to form discrete cell clusters or colonies, which can be identified and evaluated by light microscopy and individual colonies can be plucked out to be analysed (StemCell Technologies, 2004).

Wulf *et al.* used haematopoietic methylcellulose media in order to investigate side population (SP) cells sorted from murine liver (Wulf et al., 2003). $2x10^4$ cells/well were used and the colony growth was scored at days 14 and 21. $2x10^4$ hepatic SP cells generated 5.7±2.9 colonies in haematopoietic media supplemented with cytokines. With TGF-α, EGF and nicotinamide, only slight proliferation was seen suggesting an inhibitory effect of hepatocytic growth factors on early progenitor growth. The colonies were in general densely growing round cells (similar to colonies from bone marrow derived SP cells) and around 30% had a mixture of polygonal adherent cells and clusters of semi-adherent small cells and large cells. The cells were found to express hepatocyte (FAH and albumin) and haematopoietic markers (CD45) (Wulf et al., 2003).

6.2 Hypothesis

The hypotheses of this chapter were:

1) Non-parenchymal cells expressing stem cell surface markers can be isolated from human liver explants.
2) Cells sorted by stem cell markers are enriched in progenitor cells and will form colonies *in vitro*.

6.3 Aims

The aims of this chapter were to:

1) Sort putative stem cells/progenitors from non-parenchymal cells isolated from human explant livers using surface markers.
2) Culture different fractions of sorted cells to investigate colony formation *in vitro*.

6.4 Methods

6.4.1 Starting material – Non-parenchymal liver samples

The non-parenchymal cells were isolated, frozen and thawed from human explant livers as described in *Chapter 2 General Methods*.

Table 6-1 Non-parenchyml cell samples used for cell sorting experiments.

Disease aetiology	Marker	Method
Cryptogenic	CD117	FCMCS
sub-fulminant	CD117	MACS
sub-fulminant	CD133	MACS
ALD	c-met	MACS

6.4.2 Positive controls

6.4.2.1 Weri-Rb-1

Weri-Rb-1 cells express CD133 and were used as a control for experiments assessing this antigen.

6.4.2.2 MO7e

MO7e cells express CD117 and were used as a control for experiments observing this antigen.

6.4.2.3 PBMCs Peripheral blood monocytes (PBMCs)

The cells were used as positive controls for markers CD34 and CD45. For preparation of PBMC, please refer to *Chapter 2 General Methods*.

6.4.2.4 Mobilised PBMCs

Cells were provided by Mark Lowdell and Fiona O'Brien, Department of Haematology, UCL and Royal Free Medical School. Mobilised PBMCs were used for CD34 sorting experiments.

6.4.3 Flow cytometric cell sorting (FCMCS)

The experimental set-up and use of the MoFlo high speed cell sorter (DakoCytomation, Fort Collins, Colorado) was performed by Dolorez Martinez Garcia, The Wolfson Institute for Biomedical Research, UCL.

6.4.3.1 Preparing the cells for the sorter

Thawed cells were lymphoprepped and resuspended in FCMCS buffer (PBS [with Magnesium chloride 0.1g/L and Calcium chloride 0.133g/L] supplemented with 10% FCS). The cells were stained with the antibodies (CD34-APC, CD45-FITC, CD133-PE and CD117-PC5) for 15min at room temperature and washed in FCMCS buffer. The cells were filtered through a 30µm mesh before being acquired by the sorter. For specific antibody concentrations and labelling volumes please refer to *Chapter 2 General Methods*.

6.4.3.2 Collecting the sorted fractions

The $CD117^{+}$ and $CD117^{neg}$ cell fractions were collected from the MoFlow sorter. The method of separation is outlined below. Firstly the sample stream of the flow cytometer containing the cells is passed through a laser beam and analysed, as for standard FCMCS analysis. An oscillating nozzle tip then breaks down the sample stream into droplets containing the cells. When the cell of interest passes through the laser the specific droplet is identified and an electric charge (positive or negative) is applied to the stream. When the droplet passes through the deflection plates carrying a high voltage, the droplet will be attracted to one of the plates depending on the charge it was given. The different streams of droplets are then collected (Nicoll, 2004).

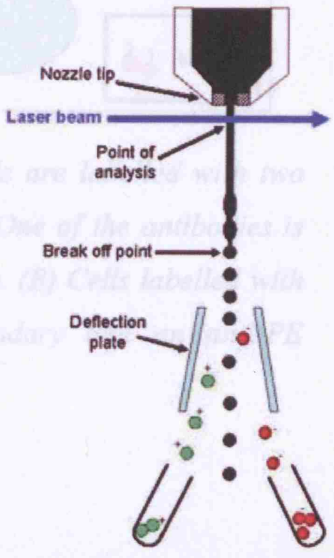


Fig. 6-1 Cell sorting by FCM. The cells are passed in a fluid stream and analysed when they pass the laser beam. The sample stream is broken into droplets which are given an electric charge depending on the analysis. The charged droplets pass through positively and negatively charged deflection plates and are attracted depending to the charge given. The different beams of droplets are then collected into tubes for further analysis. Droplets not given a charge pass through the deflection plates undeflected (Nicoll, 2004).

6.4.3.3 Culture and analysis

The sorted cells were cultured in PM and PM/HS-5 media. Cultured cells were analysed for mRNA expression using the cell-to-cDNA method. Please refer to *Chapter 2 General Methods* for detailed methods.

6.4.4 Magnetic cell sorting (MACS)

6.4.4.1 Preparing the cells for the sorter

Thawed cells were lymphoprepped (for lymphoprep methodology please refer to *Chapter 2 General Methods*) and resuspended in MACS buffer (PBS supplemented with 0.5% BSA and 2mM EDTA). The cells were incubated with a primary antibody conjugated to a super-paramegnetic microbead [Fig. 6-2 A] or a sandwich system with primary antibody, secondary antibody linked to PE and an anti-PE bead [Fig. 6-2 B]. In order analyse the sorted fractions by flow cytometry, samples with directly conjugated micro-beads were stained with fluorochrome linked antibodies directed to another epitope of the same antigen. The cell fractions were washed with MACS buffer between each staining step. Table 6-2 summarises the antibodies and microbeads used in this chapter. Incubation times and volumes were according to manufacturers instructions, please refer to *Chapter 2 General Methods*.

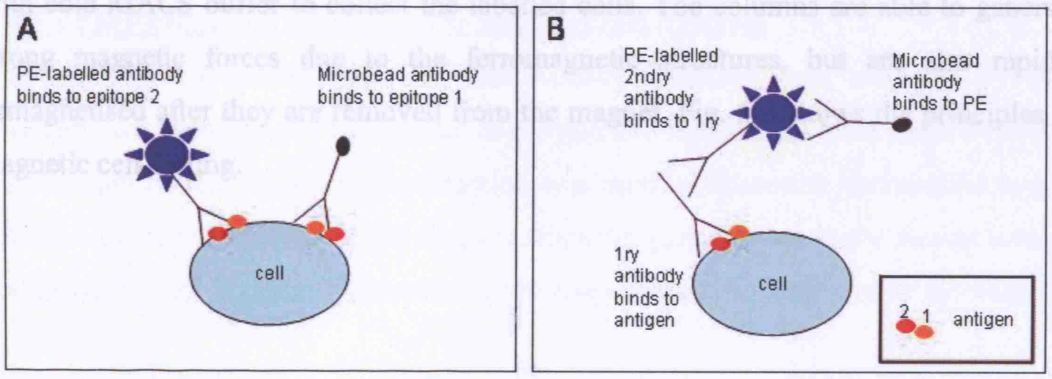


Fig. 6-2 Staining the cells for magnetic cell sorting. (A) Cells are labelled with two antibodies directed to different epitopes of the same antigen. One of the antibodies is conjugated to a microbead and the other to a PE fluorochrome. (B) Cells labelled with an antigen specific primary, followed by PE-linked secondary and an anti-PE microbead.

Table 6-2 Antibodies and microbeads used for magnetic cell sorting.

Antigen	Microbead	2ndry antibody	FCMCS antibody
CD117	anti-CD117	-	anti-CD117/2 PE
CD133	anti-CD133	-	anti-CD133/2 PE
c-met	anti-PE	anti-IgG PE	-

6.4.4.2 Sorting

Sorting columns (composed of a ferromagnetic matrix) were placed onto a permanent MACS magnet (Miltenyi biotech cat no 130-042-102) and prepared with cold MACS buffer. LS columns (Miltenyi biotech cat no 130-042-401) and Large Cell columns (Miltenyi biotech cat no 120-000-474) were used for the first separation and any further sorting was done on MS columns (Miltenyi biotech cat no 130-042-201). Stained cells were resuspended in cold MACS buffer and placed onto the column through a 30μm pre-separation filter (Miltenyi biotech cat no 130-041-407). The effluent was collected and analysed. The effluent will contain cells that were not labelled with the microbeads, i.e. the negative fraction. The cells stained with the microbead antibodies will be retained in the column due to the magnetic field of the magnet. Once the column has been washed to remove any unlabelled cells, it is removed from the magnet and flushed with cold MACS buffer to collect the labelled cells. The columns are able to generate strong magnetic forces due to the ferromagnetic structures, but are also rapidly demagnetised after they are removed from the magnet. Fig. 6-3 shows the principles of magnetic cell sorting.

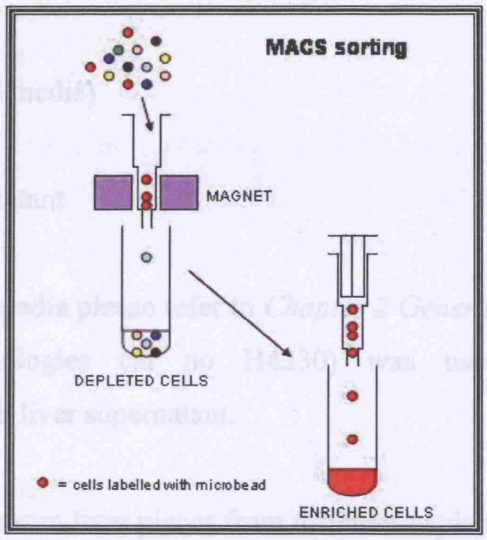


Fig. 6-3 Magnetic cell sorting. Cells are labelled with antigen specific microbeads and put through a column attached to a magnet. Unlabelled cells run through the column (collected as depleted cells) whereas the labelled cells remain in the column. The column is removed from the magnet and flushed with buffer to collect the labelled cells (collected as enriched cells).

To increase the purity of the enriched cells, two columns may be used in succession. The positive fraction from the first sort is placed onto a second a column and the positive cells collected. It is important to note that although this method greatly increases the purity of the sorted population, it also decreases the yield as positive cells are also lost in the second 'depleted fraction'.

6.4.4.3 Flow cytometric analysis of the MACS sorted fractions

The unlabelled, unsorted, negative and positive fractions were analysed by flow cytometry to determine the enrichment of the positive fraction. R1orR2 gates were used for the analysis and the unlabelled fraction was used to determine background events. The MACS microbeads are about 50nm in diameter (comparable to the size of a virus) and therefore should not influence the scatter properties of the cells in flow cytometry.

6.4.4.4 Culture of sorted fractions

The beads are biodegradable (made of iron oxide and polysaccharide) and should not affect cell viability and function. Very rare cells (down to 1 in 10^8) can be selected and cultured directly after selection. The unsorted, negative and positive fractions were cultured in order to investigate colony formation.

The following conditions were used for culture:

1) PM
2) PM + 20ng/ml HGF and 10ng/ml EGF
3) PM/HS-5 (1:1 PM and HS-5 conditioned media)
4) Methylcellulose culture
5) Methylcellulose culture with liver supernatant

For HS-5 culture and collection of conditioned media please refer to *Chapter 2 General Methods*. Methylcellulose (StemCell Technologies cat no H4230) was used supplemented with PM additives or together with liver supernatant.

The liver supernatant was prepared using snap frozen liver pieces from different explant livers. A selection of different liver disease aetiologies and patients was selected. The liver was homogenised with media using a grinder for a concentration of 50mg tissue/300μl media. The smashed up tissue was incubated for 4hrs at 4°C. The sample was centrifuged at 800g for 5 min to remove liver debris and the supernatant was further centrifuged at 150 000g for 1hr to remove cell debris. The supernatant was filter-sterilised and frozen at -80°C. For use the supernatant was thawed and 300μl liver supernatant was used together with 800μl methylcellulose, 100μl PM additives and 100μl media containing the cells. In pure methylcellulose cultures the liver supernatant was substituted with media.

6.5 Results

6.5.1 FCMCS sort

6.5.1.1 Sorting for CD117

The flow cytometric analysis of the cells prepared to be sorted revealed that no clear $CD133^+$ cells were observed and therefore $CD117^+$ cells alone were sorted instead of $CD117^+$ $CD133^+$ cells. Furthermore, the analysis showed only 0.3-0.4% of the cells to be $CD117^+$, when ideally at least 1% is expected. When cell fractions go below 1%, the machine gets less accurate in the sorting.

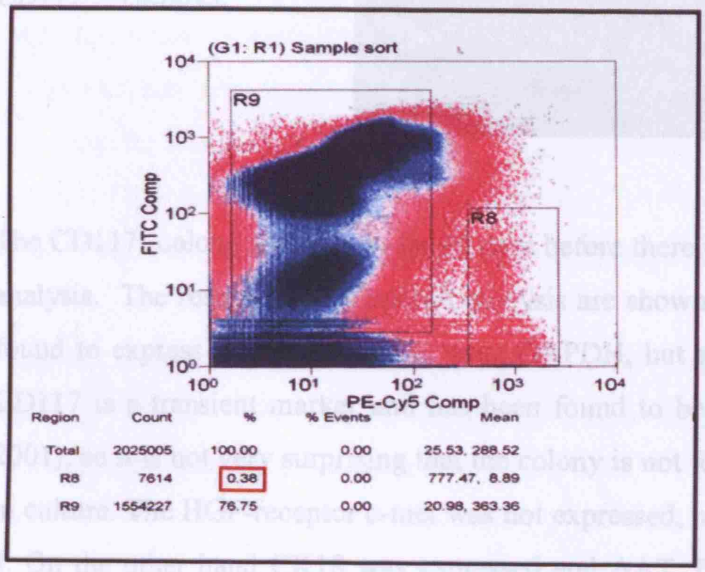


Fig. 6-4 FCM analysis with CD117-PC5 [x-axis] and CD45-FITC [y-axis]. $CD117^+$ cells are found in gate R8 and consists of 0.38% of the analysed population [red box]. These gates were used for the FCMCS sort of $CD117^+$ cells.

$1.075x10^8$ non-parenchymal cells from a cryptogenic liver explant were thawed. $1.3x10^4$ cells were recovered in the $CD117^+$ positive fraction, representing 0.012% of the thawed cells and 0.043% of the lymphoprepped cells. Two wells, one with PM and one with PM/HS-5 was set up with $6x10^3$ cells each. The remaining $1x10^3$ cells were used for mRNA analysis. Extra wells were set up for the $CD117^{neg}$ fraction of both culture conditions.

6.5.1.2 Culture and analysis of the sorted fraction

The $CD117^+$ cells from the analyser processed for mRNA analysis proved to be unsuccessful, probably due to the low number of cells used. In culture however, on day 17 a colony was found in the well containing $CD117^+$ cells in PM/HS-5 media. No

growth was observed in the negative fraction controls with the same number of cells or the well containing $CD117^+$ cells in PM .

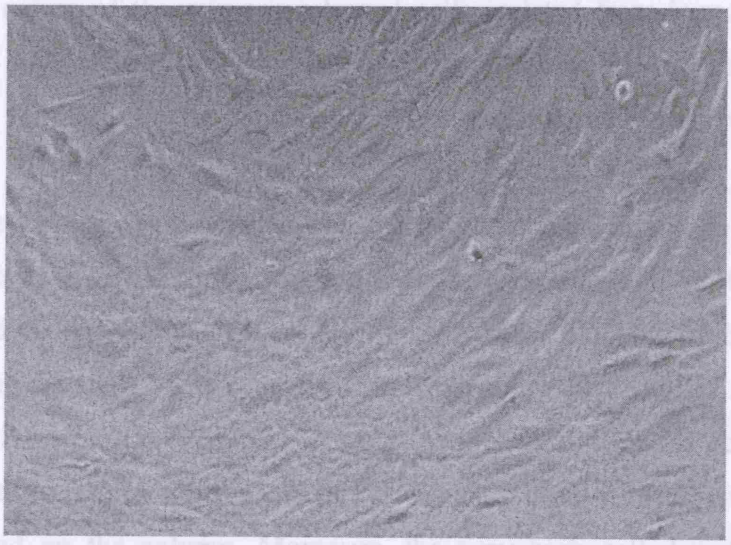


Fig. 6-5 Phase contrast light microscopy (x100) of $CD117^+$ cell colony in PM/HS-5 (day 29) with fibroblast-type morphology. No cell growth were observed in $CD117^{neg}$ cultures.

The $CD117^+$ colony was grown for 40 days before there were sufficient cells for mRNA analysis. The results for the mRNA analysis are shown in Table 6-3. The colony was found to express the housekeeping gene GAPDH, but not the sorting antigen CD117. CD117 is a transient marker and has been found to be lost in culture (Crosby et al., 2001), so it is not very surprising that the colony is not found to express it after 40 days in culture. The HGF-receptor c-met was not expressed, nor was albumin, AFP and Oct-4. On the other hand CK18 was expressed and AAT, EGF and TGF□-receptors were weakly expressed.

Table 6-3 mRNA analysis of the $CD117^+$ cell colony grown for 40 days in PM/HS-5. Cells express GAPDH, CK18 and alpha-1-antitrypsin (AAT), EGF-receptor and TGFβ-receptor are weakly expressed. No c-met, albumin (alb), alpha-fetoprotein (AFP), Oct-4 or CD117 expression is seen. The PCR gels are found in Appendix 6.

	GAPDH	CD117	c-met	EGF-R	TGF□R	alb	AAT	CK18	AFP	Oct-4
colony	+	-	-	weak	Weak	-	weak	+	-	-

6.5.2 MACS sorting

6.5.2.1 Optimising MACS

Magnetic cell sorting has been extensively used with haematopoietic and lymphoid cells. Due to the different nature of the non-parenchymal cells isolated from human liver, the standard protocol suggested by the manufacturer was optimised to take into account cell concentrations, viability, recovery and single-cell suspension maintenance.

A MACS sort was simulated using a non-labelled non-parenchymal cells sample that had been subjected to all steps involved in the staining procedure. A total of $7.9x10^7$ cells were thawed to be used for the sort on a LS column. To reduce the blocking of the column due to cell clumping the cells were kept at room temperature (with buffer at room temperature) during all staining procedures. Varying the temperature of the cell suspension has been found to cause clumping. Cold buffer was only used to resuspend the cells for the sorting and on the column. Moreover, the very high cell density suggested by the manufacturer for the column ($2x10^8$/ml) was diluted more than 100-fold ($1.5x10^6$/ml) and a pre-separation filter of 30μm was used.

Viability remained high throughout the protocol. The cells were 79% viable after thawing, 90% after the lymphoprep and 85% after the staining procedure and sorting. The recovery after the LS column was around 56%.

6.5.2.2 MACS sorting efficiency

6.5.2.2.1 CD34 from mobilised PBMCs

MACS sorting efficiency was assessed using CD34-sorting from mobilised PBMCs. A total of $2.2x10^7$ cells were thawed and sorted on a LS column followed by an MS column. The original sample contained 1.65% $CD34^+$ cells and was enriched to contain 92.29% $CD34^+$ cells [Fig. 6-6]. The first effluent contained 0.2% $CD34^+$, whereas the second effluent contained 37.12% $CD34^+$ cells. The positive fraction after the first column normally contained around 43% positive cells. Viability remained high (>85%) throughout the experiment.

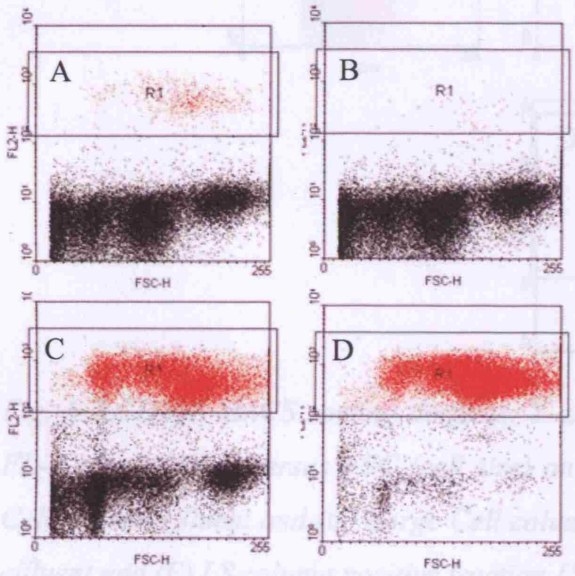


Fig. 6-6 CD34 MACS sorting dotplots. Y-axis showing CD34 positive events in the FL-2 channel (PE) versus FSC (cell size) on the x-axis. Cells in R1 are $CD34^+$. (A) unsorted fraction with a small but distinct $CD34^+$ population [1.65%] and, (B) effluent containing very few $CD34^+$ cells [0.20%]. (C) Second effluent showing a relatively large amount of $CD34^+$ cells [37.12%] and (D) the positive fraction after two columns [92.29%].

6.5.2.2.2 CD117, CD133 and c-met from non-parenchymal liver cells

Non-parenchymal cells were MACS sorted to investigate enrichment of the positive fraction. Two different columns were compared, an LS column and a Large Cell column. Because yield was considered more important than purity, only one column was used for the sorts. The same non-parenchymal samples were used for this analysis as for the culture experiments.

$CD117^+$ cells were enriched from less than 1% to 69% (large cell column) and 58.88% (LS column). Fig. 6-7 shows the dotplots for the sorted fractions. The positive cells sorted with the Large Cell column were a clearer cluster than those sorted with the LS column. The Large Cell column was used for the culture experiments.

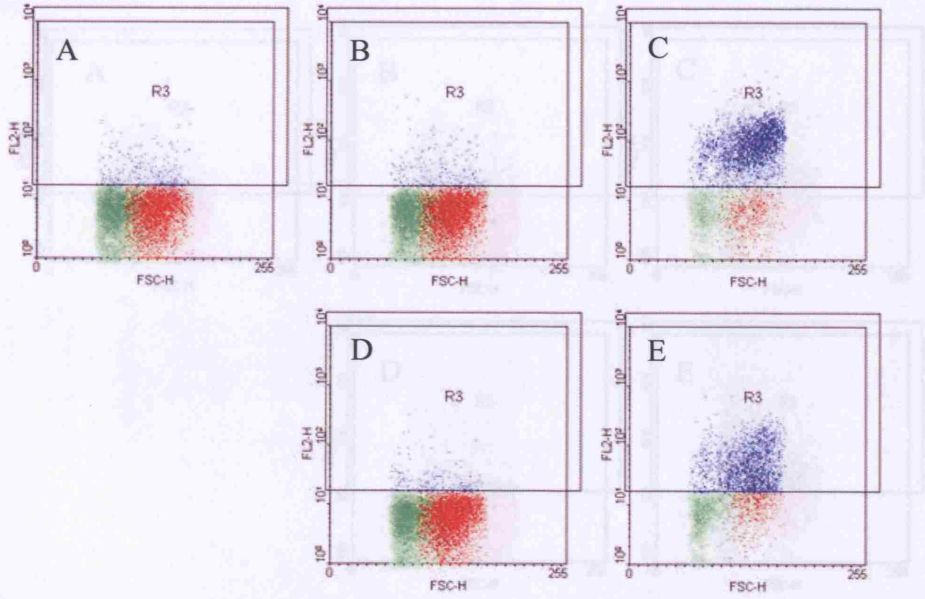


Fig. 6-7 CD117 MACS sorting dotplots. Y-axis showing CD117 positive events in the FL-2 channel (PE) versus FSC (cell size) on the x-axis. (A) Unsorted cells, (B) Large Cell column effluent and (C) Large Cell column positive fraction [69%]. (D) LS column effluent and (E) LS column positive fraction [58.88%].

$CD133^+$ cells were enriched from less than 1% to 35.89% (large cell column) and 40.19% (LS column). Fig. 6-8 shows the dotplots for the sorted fractions. The positive fractions are not as clear as with the CD117 sort, but clearer positive cells are observed with the LS column. The LS column was used for the culture experiments.

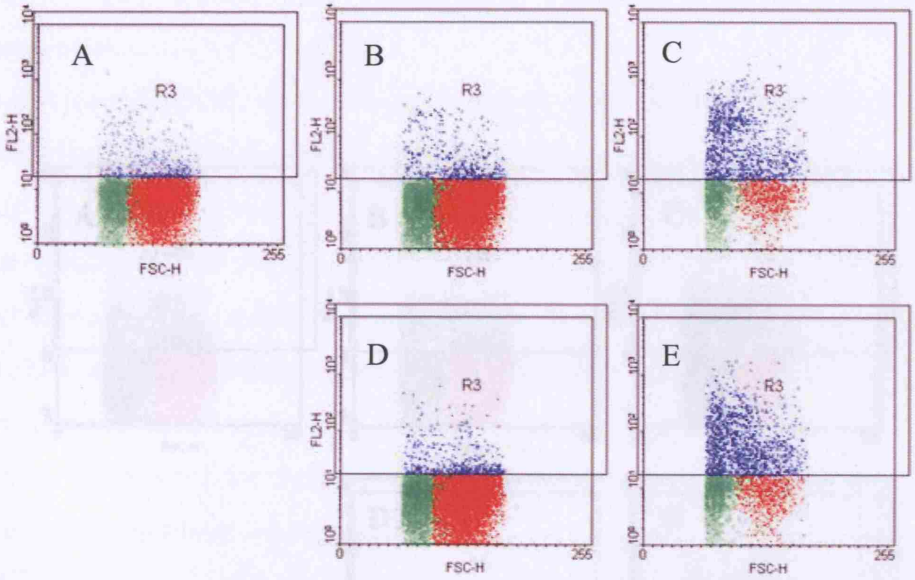


Fig. 6-8 CD133 MACS sorting dotplots. Y-axis showing CD133 positive events in the FL-2 channel (PE) versus FSC (cell size) on the x-axis. (A) Unsorted cells, (B) Large Cell column effluent and (C) Large Cell column positive fraction [35.89%]. (D) LS column effluent and (E) LS column positive fraction [40.19%].

C-met^{+} cells were enriched from 2% to 52.07% (large cell column) and 57.01% (LS column). Fig. 6-9 shows the dotplots for the sorted fractions. The positive cells sorted with the LS column have more positive cells and the populations were a clearer cluster than with the Large Cell column positive fraction. The sort was not as clean as for CD117, but better than CD133. Interestingly, however, a c-met^{high} cell population was seen in both positive fractions (top part of dotplots C and D). The LS column was used for the culture experiments.

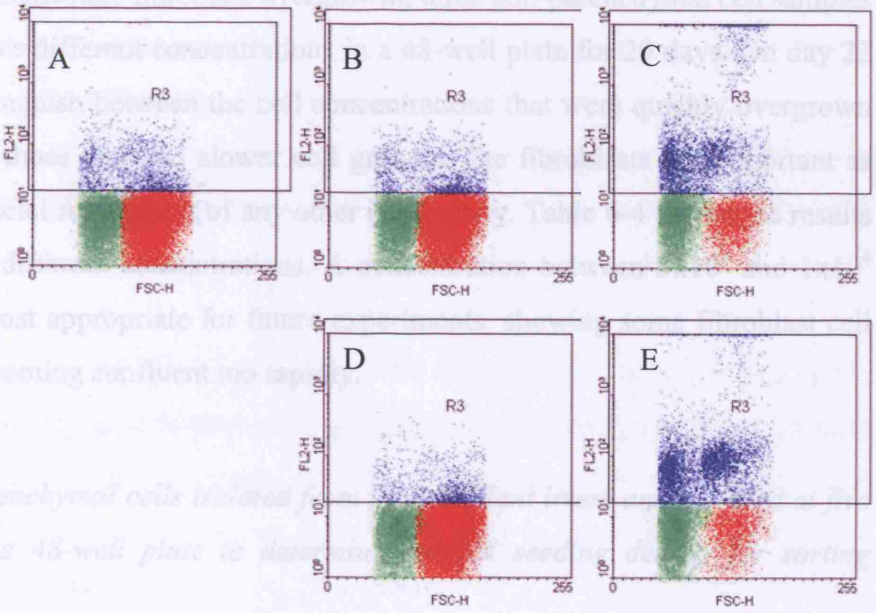


Fig. 6-9 C-met MACS sorting dotplots. Y-axis showing c-met positive events in the Fl-2 channel (PE) versus FSC (cell size) on the x-axis. (A) Unsorted cells, (B) Large Cell column effluent and (C) Large Cell column positive fraction [52.07%]. (D) LS column effluent and (E) LS column positive fraction [57.01%]. The positive cell populations are clearer with the LS column than the Large Cell column. Also note the c-met^{high} populations in both positive fractions.

For CD117 and CD133 two antibodies were used directed to different epitopes of the same antigen. One of the antibodies was linked to the microbead and the other to a fluorescent tag used for the flow cytometric analysis. This method provides an internal control for non-specific binding, because it is unlikely that both antibodies would non-specifically bind to the same cells. The cells considered positive in these sorts are probably real positive cells. For c-met, however, only one antigen specific antibody was

used, and it is therefore possible that some cells considered positive are in fact non-specific binding. Moreover, due to the 'sandwich system' with the antibodies, other background effects might be observed. It is important to take this into account when analysing the positive cells. On the other hand, the c-met^{high} population is likely to be real due to the large shift of the cluster compared to the negative cells.

6.5.2.3 Culture of sorted fractions

6.5.2.3.1 Optimising culture conditions

In order to determine a seeding density of non-parenchymal cells that would allow cell growth, but avoid immediate fibroblast overgrowth, three non-parenchymal cell samples were cultured at five different concentrations in a 48-well plate for 29 days. On day 22 it was easy to distinguish between the cell concentrations that were quickly overgrown by fibroblasts and those that had slower cell growth. The fibroblasts are important as they may be beneficial for support of any other cell colony. Table 6-4 shows the results on day 22 for the different concentrations. A concentration between $5x10^4$ and $1x10^4$ was found to be most appropriate for future experiments, showing some fibroblast cell growth without becoming confluent too rapidly.

Table 6-4 Non-parenchymal cells isolated from three explant livers and cultured at five concentrations in a 48-well plate to determine optimal seeding density for sorting experiments.

	PSC	ALD	PBC
$1.5x10^5$	partly confluent	confluent	confluent
$5x10^4$	few cells	confluent	confluent
$1x10^4$	few cells	partly confluent	partly confluent
$5x10^3$	no cells	few cells	partly confluent
$1x10^3$	no cells	few cells	few cells

Methylcellulose cultures were set up at three concentrations ($1x10^5$/ml, $1x10^4$/ml and $1x10^3$/ml) for three different non-parenchymal cell samples. No growth or cell colonies were observed in two of the samples, but some fibroblast growth was observed at $1x10^5$/ml in one of the samples. The optimal cell concentration was thought to be less critical due to low level of fibroblast overgrowth. Because the cells have no cell-to-cell

contact in methylcellulose, it is unlikely that a low seeding density would cause problems.

Methylcellulose media supplemented with liver supernatant was set up as normal methylcellulose cultures. However, analysis was not possible due to the opaque appearance of the methylcellulose together with the liver supernatant, where the wells could not be observed under a light microscope.

6.5.2.3.2 Culture of $CD117^+$ cells

$1.957x10^8$ non-parenchymal cells isolated from a sub-fulminant liver explant were thawed. $1.68x10^5$ cells were recovered in the positive fraction, representing 0.086% of the thawed cells and 0.387% of the lymphoprepped cells. Two wells for each culture condition were set up at a seeding density of $2.5x10^4$. Remaining cells ($1.8x10^4$) were placed in methylcellulose culture. Extra wells were set up for unsorted and $CD117^{neg}$ fractions of each culture condition and methylcellulose.

The results of the culture of CD117 sorted cells are seen in Table 6-5. The results are shown as 'wells with cell growth/total wells set up'. No cell growth was observed with the $CD117^+$ fraction in liquid culture. However, fibroblast-type cell growth was observed in methylcellulose, whereas no growth was seen in the unsorted or negative fractions in methylcellulose.

Table 6-5 CD117 sorted fractions in different culture conditions at $2.5x10^4$ cells/well. Results expressed as 'wells with cell growth'/'total wells set up'. Methylcellulose colony in $CD117^+$ fraction was replated but did not yield any more colonies.

	PM	PM/GFs	PM/HS-5	methylcellulose
$CD117^+$	0/2	0/2	0/2	1/1
$CD117^{neg}$	0/5	1/5	2/5	0/2
unsorted	1/5	1/5	2/5	0/2

Fig. 6-10 shows the colony observed in methylcellulose culture of $CD117^+$ cells. The colony was replated after 3 weeks of culture into normal culture media and new methylcellulose was added to the original well due to dryness of the original methylcellulose. Unfortunately the replated cells did not attach or grow. The cell growth found in the $CD117^{neg}$ and unsorted cultures had a fibroblast-type morphology [Fig. 6-11].

Fig. 6-10 Phase contrast light microscopy (x100) of $CD117^+$ cell colony in methylcellulose culture (day 16) with fibroblast-type morphology. No cell growth were observed in $CD117^{neg}$ and unsorted fractions.

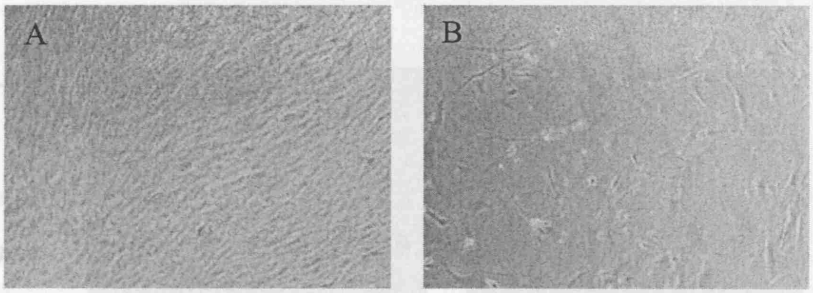


Fig. 6-11 Phase contrast light microscopy (x100) of fibroblast-type colonies found with culture of (A) $CD117^{neg}$ cells and (B) unsorted cells in liquid culture.

6.5.2.3.3 Culture of $CD133^+$ cells

$7.44x10^7$ non-parenchymal cells isolated from a sub-fulminant explant liver were thawed. $2.73x10^5$ cells were recovered in the positive fraction, representing 0.366% of the thawed cells and 1.078% of the lymphoprepped cells. Three wells for each culture condition were set up at a seeding density of $2.5x10^4$. Remaining cells ($2.4x10^4$/well)

were placed in two wells in methylcellulose. Extra wells were set up for unsorted and $CD133^{neg}$ fractions of each culture condition and methylcellulose.

The results for CD133 culture are shown in Table 6-6. No colonies were observed in methylcellulose culture with any of the fractions. Fibroblast-type growth was observed in one $CD133^{+}$ well cultured with PM/HS-5. However, similar growth was seen in $CD133^{neg}$ and unsorted cultures. Fig. 6-12 shows cell growth of the different fractions in PM/HS-5 media. Some fibroblast-type growth was also seen in PM and PM/GFs media with the negative and unsorted fractions, but at a lower frequency.

Table 6-6 CD133 sorted fractions in different culture conditions at $2.5x10^4$ cells/well. Results expressed as 'wells with cell growth'/'total wells set up'. No colonies with interesting morphologies were observed. Fibroblast-type growth was seen in one $CD133^{+}$ well cultured with PM/HS-5 media. This type of growth was also seen in $CD133^{neg}$ and unsorted culture.

	PM	PM/GFs	PM/HS-5	methylcellulose
$CD133^{+}$	0/3	0/3	1/3	0/2
$CD133^{neg}$	0/6	1/6	3/6	0/2
unsorted	1/6	0/6	3/6	0/2

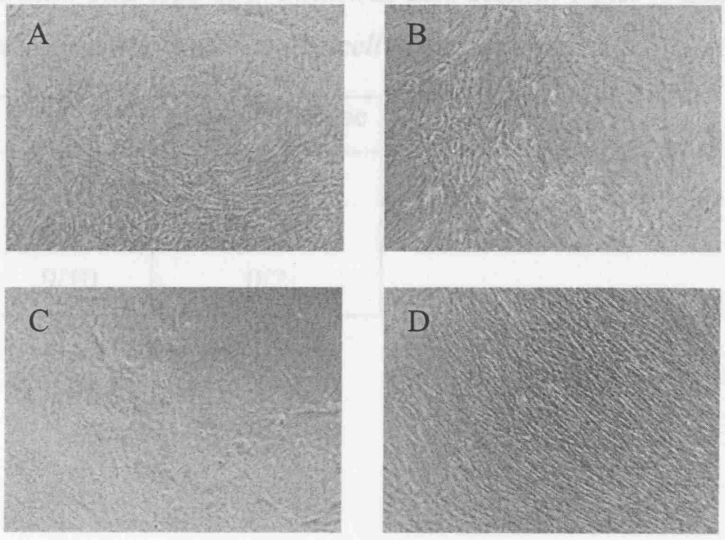


Fig. 6-12 Phase contrast light microscopy (x100) of fibroblast-type colonies found in PM/HS-5 culture with (A) $CD133^{+}$ cells [day 23], (B) $CD133^{neg}$ [day 15], (C) unsorted [day 29] and (D) unsorted [day23] cells.

6.5.2.3.4 Culture of c-met$^+$ cells

$2.18x10^8$ non-parenchymal cells isolated from an ALD explant were thawed. $18.75x10^5$ cells were recovered in the positive fraction, representing 0.862% of the thawed cells and 1.774% of the lymphoprepped cells. Ten wells for each culture condition were set up at a seeding density of $2.5x10^4$. An additional two wells in each culture condition was set up at $1.5x10^5$ cells/well. Remaining cells ($1x10^5$/well) were placed in two wells in methylcellulose. The same number of wells was set up for all fractions.

The results of the culture of c-met sorted cells are seen in Table 6-7 and for the higher seeding density in Table 6-8. The results are shown as 'wells with cell growth/total wells set up'. No cell growth was observed in PM and PM/GFs culture conditions for c-met$^+$ cells. Fibroblast-type growth was seen in 4/10 wells with PM/HS-5 culture media. This was, however, also observed in the c-met^{neg} and unsorted fractions [Fig. 6-13]. Some cell growth was also seen in PM and PM/GFs media in the negative and unsorted fractions, again at a lower frequency than PM/HS-5. At the higher seeding density most wells became confluent. In methylcellulose, c-met^{neg} fractions contained cell growth [Fig. 6-14]. This was similar to the CD117$^+$ colony in methylcellulose.

Table 6-7 C-met sorted fractions in different culture conditions at $2.5x10^4$ cells/well. Results expressed as 'wells with cell growth'/'total wells set up'. No colonies with interesting morphologies were observed. Fibroblast-type growth was seen in c-met$^+$ wells cultured with PM/HS-5 media. This type of growth was also seen in c-met^{neg} and unsorted culture. Cell growth was seen in c-met^{neg} methylcellulose cultures.

	PM	PM/GFs	PM/HS-5	methylcellulose
c-met$^+$	0/10	0/10	4/10	0/2
c-met^{neg}	2/10	3/10	8/10	2/2
unsorted	2/10	6/10	9/10	0/2

Table 6-8 C-met sorted fractions in different culture conditions at $1.5x10^5$ cells/well. Results expressed as 'wells with cell growth'/'total wells set up'. No colonies with interesting morphologies were observed. Fibroblast-type growth was seen in most wells at the high seeding density.

	PM	PM/GFs	PM/HS-5
c-met$^+$	0/2	2/2	2/2
c-met^{neg}	2/2	2/2	2/2
unsorted	1/2	2/2	2/2

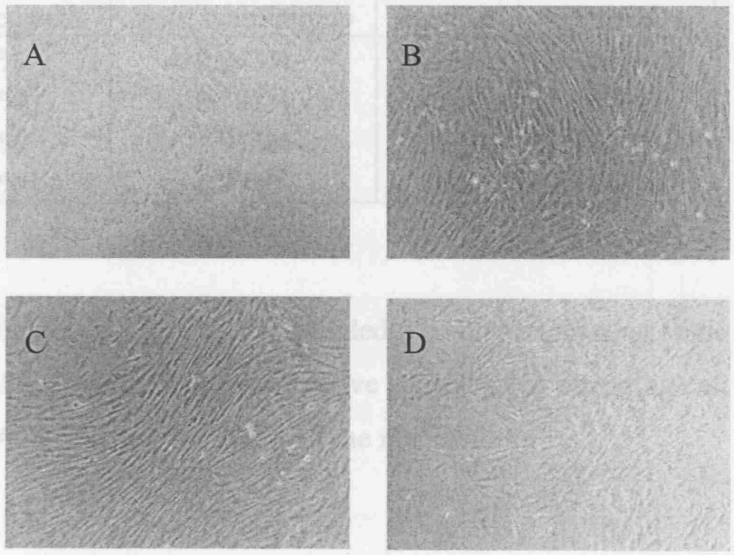


Fig. 6-13 Phase contrast light microscopy (x100) of fibroblast-type colonies found in PM/HS-5 culture with (A) c-met$^+$ cells [day 22], (B) c-met$^+$ [day 29], (C) c-met^{neg} [day 22] and (D) unsorted [day 29] cells.

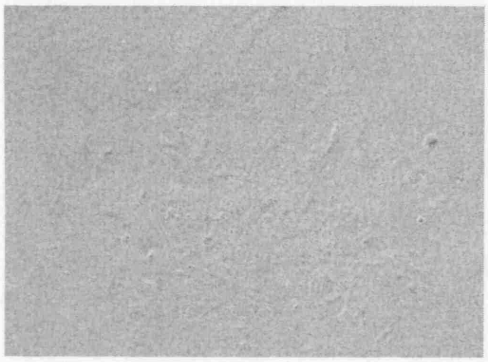


Fig. 6-14 Phase contrast light microscopy (x100) of c-met^{neg} cell colony in methylcellulose culture (day 22) with fibroblast-type morphology.

6.5.2.4 Summary

Table 6-9 summarises the percentages of the sorted cells and the percentages of positive cells isolated from thawed and lymphoprepped cells (by cell counts) and the percentage of positive cells in the starting population and the positive fraction after the sort (by flow cytometry).

Table 6-9 The percentage of positive cells isolated calculated from number of cells thawed and the number of cells after the lymphoprep gradient. The percentage of positive cells analysed by flow cytometry in the original starting population and in the positive fraction following the sort.

	from thawed cells	from lymphoprep	original	+ve fraction
FCMCS CD117	0.012%	0.043%	0.38%	n/a
MACS CD117	0.086%	0.387%	<1%	69%
MACS CD133	0.366%	1.078%	<1%	40.19%
MACS c-met	0.862%	1.774%	2%	57.01%

Cell culture of the $CD117^+$ cells isolated by FCMCS yielded a colony expressing some liver cell markers at a low level and with a high proliferative capacity. No intresting cell growth was observed in the MACS fractions with any of the markers.

6.6 Discussion

6.6.1 Cell sorting of non-parenchymal cells

The markers chosen for sorting were CD117 and CD133, haematopietic stem cell markers, which have also been associated with putative liver stem cells (Craig et al., 2004b). CD117 was also observed on some cells in the npcRTx colony and by flow cytometry distinct, although very small, populations of $CD117^+$ and $CD133^+$ cells were observed. C-met, the HGF-receptor, was also used. C-met has been identified to be important in foetal liver development and expressed on liver stem cells isolated from foetal liver (Suzuki et al., 2002; Suzuki et al., 2004). The colonies npcRTx and W3, W4 and W5 all expressed c-met by RT-PCR and immunocytochemistry, as did the colony isolated by Selden *et al.* by RT-PCR (Selden et al., 2003).

$CD117^+$, $CD133^+$ and $c\text{-}met^+$ cells were successfully sorted from non-parenchymal cells isolated from explant livers by MACS. Although very low numbers of cells in the starting population (<2%) was observed, a purity of 40% - 70% after the sort was acquired. The results for the non-parenchymal cell sorts were comparable to the positive control sort of CD34 from mobilised PBMC. The purity of the non-parenchymal positive fractions might be further improved by using a second sort, but were not investigated due to the trade-off loss of positive cells in the second sort as shown for CD34 cells. Unfortunately there was no comparison between the purity of $CD117^+$ cells sorted by FCMCS and MACS, due to the very low yields of positive cells.

The number of positive cells acquired was lowest for CD117, both in total and as a percentage of the lymphoprepped cells (0.043% from FCMCS and 0.387% from MACS). The yield of CD117 from FCMCS and MACS was not comparable due to the different liver explants used. The yield of $CD133^+$ cells was higher but still only around 1% of the lymphoprepped cells. The yield of c-met was around 2%, and due to the higher number of cells started with, the sort generated a higher number of cells. The data loosely corresponds to the data collected in *Chapter 5* for the percentage of positive cells expressing the markers, although CD117 and CD133 analysis were not very accurate in this chapter because no extra gates were used for the FCMCS analysis of the MACS fractions.

6.6.2 Culture of sorted fractions

The different MACS sorted populations were successfully cultured *in vitro*, although no interesting colonies were observed. Fibroblast contamination was observed at high seeding densities ($1.5x10^5$ used for c-met), and in wells containing PM/HS-5 media. The conditioned media collected from HS-5 (bone marrow derived stromal cell line) cell culture was thought to provide growth support for fibroblastic cells in the non-parenchymal cells, in addition to the cytokines (used for promoting haematopoietic stem cells in culture). Therefore more fibroblast contamination was seen in these wells. Less fibroblast contamination was observed in methylcellulose media even at higher cell densities. However, no colonies as described by Wulf *et al.* were observed (Wulf et al., 2003). The methylcellulose assay would have provided an ideal starting point for any cell lineage derived from adult liver stem cells as the cell colonies would have been clonal and could have been plucked out for analysis or further culture.

The cell colony found in the FCM sorted $CD117^+$ cell fraction was investigated further due to its high growth potential (starting with $6x10^3$ cells) that was not seen in any control well with similar seeding density. The mRNA analysis of the colony after 40 days in culture showed some weak liver cell marker expression (AAT, EGF-receptor and TGFβ-receptor). CD117 expression was probably lost due to long-term culture. Not enough cells or cDNA was available for a more extensive investigation. However, the mRNA expression profile available showed a different profile to the npcRTx colony in *Chapter 3* as well as W3, W4 and W5 isolated in *Chapter 4*. The other colonies all expressed c-met and Oct-4, which the sorted colony did not. The other colonies, as well as, the sorted cells expressed GAPDH, EGF-receptor, TGFβ-receptor and CK18 but none of the colonies expressed albumin. AAT expression was observed in the sorted colony as well as the W3-5, but not by npcRTx. It is very interesting to note these differences between the isolated putative liver progenitor cell colonies.

CD117 sorting of human liver cells has been shown by Crosby *et al.* to yield cells of biliary epithelial cell lineage when cultured in media promoting their differentiation (Crosby et al., 2001). Even though our experiments using PM media, which is thought to support hepatocyte growth, was unsuccessful, $CD117^+$ cell expansion in biliary epithelial cell growth media could be experimented on. However, the seeding density or purity of the positive cells in their experiments was not reported on, which may be important for the cell culture. To further investigate other methods for the generation of

liver stem cell and progenitor colonies, growth factors (liver specific as well as stem cell supporting) and liver supernatant derived from diseased livers may be utilised in culture (Jang et al., 2004). Jang *et al.* showed that $CD34^+$ cells isolated from bone marrow of adult mice could be differentiated to hepatocyte-like cells $CK18^+CD45^{neg}alb^+$ by culturing them with injured liver or liver supernatant. The cells were separated from the liver by a transwell, to exclude the possibility of fusion. In our experiments, liver supernatant and methylcellulose caused the culture system to be opaque and unsuitable for light microscopy. The use of a transwell system could be utilised to circumvent this problem.

In order to reduce fibroblast contamination, purer populations may be sorted by using two or more MACS columns. Anti-fibroblast antibodies exist that may be used for fibroblast depletion before the positive selection. Fibroblast growth may also be inhibited by specific media and by using replating methods (moving all unattached cells into a new culture well) as fibroblasts seem to be one of the first cell types to attach.

6.6.3 Future work

The work in this chapter demonstrates the possibility of non-parenchymal cell sorting by MACS and FCMCS. For FCMCS a larger and/or well defined cell population is necessary to ensure a clean and accurate sort. However, MACS enrichment of up to 70 times was seen (CD117). These sorting methods can be used for analysing the putative stem cell fractions (using markers already analysed in this chapter and other surface antigens, e.g. ABCG2) further by either different cell culture settings or by characterising the different fractions.

For example, the stem cell/liver progenitor markers of the sorted fractions can be analysed by using microarray technology. A large number of markers are now known to be associated with stem cells, liver stem cells and liver progenitors. The expression levels of the different markers could be investigated in the positive, negative and unsorted fractions. Due to the small number of positive cells, this has been difficult to achieve in the past. However, because of recent advances in microarray technology which now allow less starting material (due to extra amplification steps) and more structured chips (only containing genes known to be associated with stem cells), the method has become even more powerful (except for the cost).

Sorting can also be useful for other types of experiments. For example, Lavon *et al.* introduced a fluorescent marker, eGFP, under the control of albumin promoter into ES cultures (Lavon et al., 2004). The cultures were then cultured in differentiating conditions (conditioned media from cultured adult hepatocytes was most effective) and GFP expressing cells isolated using FCMCS. Hepatic-like cells were thus isolated from human ES cell cultures.

Chapter 7 - Discussion

7.1 Back to basics

The aim of this thesis was to investigate liver stem/progenitor cell potential for therapeutic use in liver disease. In order to achieve this, the aims were to isolate proliferating populations of putative liver stem cells, to characterise and culture them long-term and make them differentiate. Moreover, possible surface stem cell markers were hoped to be identified, which would facilitate the isolation of these cells.

7.2 How far did we get?

In *Chapter 3* a colony (npcRTx) with epithelial-like morphology was isolated from a re-transplant liver. The cells were found to express both hepatocyte and biliary epithelial cell markers by RT-PCR (c-met, EGF-receptor, TGFβ-receptor, CYP1B1, CK18, gamma-glutamyl transpeptidase) and immunocytochemistry (CK19, CK8, CK18, c-met), as well as, stem cell markers (Oct-4 [RT-PCR] and CD117 [immunocytochemistry]). The cells were kept in culture for more than six months, but their proliferative capacity was very limited and they finally senesced. In our laboratory another cell colony has been isolated by Selden *et al.* from a sub-fulminant liver explant (Selden et al., 2003). This cell colony was kept in culture for more than 5 months, but also had limited proliferative capacity. The cells expressed hepatocyte and biliary epithelial cell markers by RT-PCR (c-met, TGFβ-receptor, CK7, CK18, CK19, AAT, albumin, gamma-glutamyl-transpeptidase biliary glycoprotein) and secreted proteins into the conditioned media (albumin and AAT). Oct-4 was also expressed. These two colonies differed by their expression of CK8 (only by npcRTx), AAT, albumin and biliary glycoprotein (only by colony isolated by Selden *et al.*).

In order to tackle senescence and proliferation, *Chapter 4* utilised colonies with an epithelial-like morphology to investigate immortalisation using hTERT transduction. Telomerase maintenance is associated with immortal cell lines and stem cells and is thought to be a 'natural' way of maintaining cells without affecting the normal cell cycle and deteriorating function (Saldanha et al., 2003b; Carpenter et al., 2003; Elwood et al., 2004). Three colonies (W3, W4 and W5) isolated from an ALD explant were successfully transduced and maintained for 12 months (W5) or 20 months (W3 and

W4). The cell colonies again were found to express hepatocyte and biliary epithelial cell markers by RT-PCR (c-met, EGF-receptor, TGFβ-receptor, CYP1B1, CK18 and also AAT) and immunocytochemistry (c-met, mixture of CK7, CK8 and CK18). The colonies had different expression patterns of gamma-glutamyl transpeptidase (only expressed by W5) and cytokeratin profiles. CK7 was expressed only by W3 and 4 (not W5), and in W4 all cells expressed CK8 whereas negative and positive cells were present for W3 and W5. All cells in W3 and W4 were found to express CK18 whereas negative and positive cells present for W5. The three different colonies were therefore different from each other, as well as, to npcRTx and the colony isolated by Selden *et al.* (Selden et al., 2003). To determine the functional properties of W3, W4 and W5, the cells were stimulated with HGF and OSM to induce hepatocyte differentiation. Unfortunately, no indication of differentiation was observed.

To undertake the time-consuming and difficult task of identifying putative stem/progenitor cell colonies by morphology, *Chapter 5* concentrated on stem cell associated surface markers that could be used to isolate enriched populations of interesting cells. Furthermore this was important to be able to isolate cell populations which were more homogenous as all colonies isolated by morphology alone had proven to yield different characteristic profiles.

The markers ABCG2 (protein pump associated with drug and Hoeachst efflux), c-met/CD49f (foetal stem cell markers) and CD117/CD133 (haematopoietic stem cell markers) were identified (Goodell et al., 1996; Suzuki et al., 2002; Craig et al., 2004b). These markers were used to analyse non-parenchymal cell samples isolated from different explant liver samples by flow cytometry. It was found that ABCG2 expression was varied between different patients, but diseased explants (both chronic and acute) were found to contain ABCG2 positive cells more readily than normal BS liver resection samples (although no significant difference was found between diseased and BS samples due to variability between patients). Because biliary epithelial cells also express ATP-transporter proteins, some of the non-parenchymal samples were analysed for HEA125 (human epithelial antigen 125), but no correlation was found which suggests the cells were not biliary epithelial cells.

Both c-met and CD49f expression were also analysed. A significantly larger proportion of c-met positive cells was observed in normal BS samples compared with diseased (chronic and acute, ALD and fulminant). This was not expected but it is possible that progenitors present in diseased adult explant livers would not express c-met at this stage or that the nature of the BS samples (resections removed due colorectal cancer) would have an implication as c-met is also a tumour marker.

However, the proportion of CD49f positive cells was found to be significantly higher in diseased (chronic and PBC, PSC) compared with normal BS samples. Integrin upregulation has been observed in human liver disease and has been found to be important in foetal development, as well as, differentiation of liver cells.

The percentage of CD117 and CD133 positive cells were too small in order to compare them between different explant aetiologies. However, clear populations of positive cells ($CD117^+$ or $CD133^+$) were observed.

In *Chapter 6* CD117, CD133 and c-met were used to sort for putative stem cell/progenitor cell populations. Using flow cytometric cell sorting to isolate CD117 positive cells yielded a colony with high growth potential expressing liver markers by RT-PCR (EGF-recpetor, TGFβ-receptor, AAT and CK18). However, the colony did not express c-met or Oct-4, which was expressed by the other colonies in *Chapter 3* and *Chapter 4*.

On the other hand, magnetic cell sorting successfully enriched the positive fractions of CD117, CD133 and c-met sorts. Unfortunately, no colonies were identified in either normal or methylcellulose culture. This does not however rule out the importance of these cell fractions, but highlights the possible restrictions of our culture systems.

7.3 What next?

In this thesis we successfully isolated and characterised putative stem colonies from diseased human liver and used hTERT transduction to prolong their lifespan. Moreover, stem cell associated markers were identified on non-parenchymal cells these markers

could be used to acquire enriched cell populations by flow cytometric and magnetic cell sorting.

However, there are important issues that still need to be assessed. Firstly, the differentiation potential of the isolated colonies was not fully assessed. HGF and OSM did not induce differentiation of W3, W4 and W5. However, the differentiation of stem cells is a complex process and it might be difficult to achieve the perfect microenvironment *in vitro*. Other growth factors, as well as, co-culture with injured liver, hepatocytes or liver supernatant may be experimented on. Moreover, a useful and informative method would be the transplantation of the cells into NOD-SCID mice with and without liver injury. This would enable analysis of both integration and function of these cells in a host animal.

Secondly, the analysis of the sorted fractions should be re-assessed. Our culture method, although useful for the isolation of colonies expressing markers of both hepatocytes and biliary epithelial cells, might not be ideal for earlier or later stem /progenitor cell growth. More rigorous methods for analysis of the putative stem cells should be investigated. Transplantation into animal models, as mentioned above and detailed profiles of the isolated populations using microarray methodology would be ideal.

Finally it seems relevant to note that whilst identification and culture of stem cells from rodent liver seem to be achieved relatively easily, there remain very few reports from human liver. Whilst some of this reflects the difficulties (practical/ethical) involved in such work, it is also possible that human liver progenitor cells are inherently scarcer and that they are less robust in culture.

The next section will re-address the question 'what are stem cells?' in order to provide new ideas for their identification and isolation.

7.3.1 What are stem cells?

Recently a large amount of work has focused on creating a common gene expression profile for stem cells. Even now, the only definition of a stem cell remains to be its ability to self-renew and differentiate into different lineages. This concept implies a common genetic signature (Ramalho-Santos et al., 2002; Ivanova et al., 2002).

Microarray and SAGE (Serial Analysis of Gene Expression) technology has been used to investigate specific stem cell populations (e.g. ES, HSCs, neural stem cells) but also to compare these profiles between each other, the different studies, between species (human and mouse) as well as the sources of stem cells (CD34 cells from bone marrow, UCB and mobilised PBMCs) with the aim of creating a gene expression definition of 'stem cellness".

Microarrays use gene chips that contain gene sequences on a glass slide. The mRNA from the sample is first labelled with a fluorescent dye and then placed onto the DNA microarray slide. The mRNA will hybridize to its complementary DNA on the microarray and leave a fluorescent tag that can be quantified. If a gene is very active, i.e. many mRNA copies, there is a stronger fluorescence than if the gene is less active. If there is no mRNA, there will be no fluorescence (National Human Genome Research Institiute, 2005).

SAGE, on the other hand, allows quantitative analysis of a large number of transcripts without the prerequisite of a hybridization probe for each transcript. Firstly, short sequence 'tags' (10-14 bp) are generated that contain enough information to uniquely identify a transcript, provided that it is derived from a defined location within that transcript. Secondly, the tags are linked to rapidly sequence multiple transcripts and the identity of multiple tags can then be revealed. The expression pattern of any population of transcripts can be quantitativley evaluated by determining the abundance of individual tags and identifying the gene corresponding to such tag (BioTeach, 2005).

Abeyta *et al.* used microarrays to compare three independently derived human embryonic stem cell lines (HSF-1, HSF-6 and H9). The majority of the genes examined were expressed in all three cell lines (52%), key genes at significant levels included *Oct-4, Sox2, Rex1, TDGF1* and *LeftyA*. However, each line also possessed a unique expression signature. The differences between the cell lines were assumed to be due to the underlying genetics of the embryos from which the lines were derived and/or inherent differences in the initial culture of each line (Abeyta et al., 2004).

Rao *et al.* have reviewed the gene expression profile studies carried out on ES cells (Rao and Stice, 2004). The similarities in different human ES cell lines are found to be

in functional ontologoies that can define pluripotency, i.e. signalling pathways (signalling molecules, secreted factors, growth factors/receptors), cell cycle regulation and transcriptional regulation. Moreover, ES cell lines cluster tightly together indicating major similarities. In H1, a human ES cell line, FGF, TGFβ/BMP and Wnt pathways were found to be important for pluripotency, with high expression of *Oct-4, LeftyA, LeftyB* and *TDGF1*. Different studies comparing multiple human ES cell lines have shown that genes such as *Nanog, GTCM-1, connexion 43, Oct-4, TDGF1, DNMT3, CRABP1, SAll2* and *GABRB3* are important. Significant overlap between mouse and human ES cell lines has been observed (Rao and Stice, 2004). It is also interesting to note that *Oct-4* expression could be identified in HepG2 cells and human liver in our studies.

Yamamoto *et al.* used mouse ES cells to induce differentiation of hepatocyte-like cells (using their differentiation system which involves FGF1, HGF, OSM) and compared these cells to undifferentiated ES cells and adult mouse liver by microarray (Yamamoto et al., 2005). 232 genes were found to change their expression levels. Up-regulated genes were mainly metabolic enzymes (*P450, alcohol dehydrogenase*) and serum proteins (*TTR, albumin and major urinary protein 1*). Downregulation was seen in glycolysis-related proteins (*phosphoglycerate kinase 1, lactate dehydrogenase 1*). Many of the remaining genes were cell growth, proliferation and physiology-associated genes. *Oct-4* and *Nanog* were also downregulated. The differentiated cells were found to be very similar to adult liver with clustering analysis (Yamamoto et al., 2005).

In order to compare embryonic and adult stem cells Ramalho-Santos *et al.* looked at mouse ES cells and neural stem cells and HSCs (Ramalho-Santos et al., 2002). The transcriptional profiles of the stem cells were first compared to differentiated cells to identify up-regulated genes, and the enriched genes were then compared between the different stem cell populations.

The analysis showed that the different stem cells were found to be enriched in markers previously known to be associated with the stem cells; for example HSC (*Cd117, Cd34, Abcg2, Notch1*), neuronal stem cells (*nestin, Sox2, EgfR, Notch1*) and ESCs (*Oct-4, Rex1, Smad4*). They found that the same 216 genes were enriched in all three types of

stem cell, and that stem cells have characteristics of cells under stress. Moreover, neural stem cells had more similarities to ES cells (61.6%) than with HSCs (24.1%).

Ivanova *et al.* generated gene expression profiles by microarray for murine foetal (foetal liver) and adult HSCs (bone marrow), human foetal HSCs and murine ES cells and neural stem cells (embryonic neurospheres) (Ivanova et al., 2002). Many new and known HSC markers (e.g. *CD117, Gata2, MDR1*) were identified, and foetal and adult HSCs were found to be very similar as more than 70% of all HSC-related gene products were expressed in both profiles. Mouse and human comparisons also showed significant co-expression (39%). A general 'stem cell profile' was investigated using murine HSCs ES cells and neuronal stem cells. A total of 283 genes were commonly enriched (Ivanova et al., 2002).

Fortunel *et al.* utilised the data from Ramalho-Santos *et al.* and Ivanova *et al.* and work from their own laboratory (ES cells, neural stem cells and retinal progenitor/stem cells) to investigate the overlap of the expression of these common stem cell genes (Fortunel et al., 2003). When the three lists of common stem cell genes were compared only one gene, alpha-6 integrin, was found on all the lists (although this overlap may be by chance also). However, when comparing ES cell profiles from the three different studies, 332 genes were found to overlap, and for neural stem cells 236 genes overlapped (Fortunel et al., 2003). The analysed fractions were therefore found to be similar but no 'stemness' genes were identified. It is possible that the commonly identified genes were not genes expressed highly compared with differentiated cells and therefore not relatively highly elevated or that the different stem cell types use different gene networks to achieve self-renewal or multipotency (Fortunel et al., 2003).

Differences have also been identified in HSCs isolated from different sources. The $CD34^{+}CD38^{neg}lin^{neg}$ cells were isolated from normal human adult bone marrow, UCB and mobilised adult PBSCs and compared with $CD34^{+}CD38^{+}Lin^{+}$ cells from each tissue (Georgantas, III et al., 2004). The microarray identified the enrichment of 81 common genes and down-regulation of 90 genes (40.1%). Bone marrow and UCB cells shared 50.4% of the gene expression profiles, UCB and PBSCs 54.9% and bone marrow and PBSC 59.7%. The genes common to all three included some genes already known to be important for HSCs, e.g. *CD117, Flt3, GATA2, GATA3, p27, HoxA5, HoxA9, CD34* and

MDR2. SAGE confirmed the expression of most of the enriched genes, but suggested that that microarrays did not detect as many as 30% of the transcripts expressed in HSCs. This might be due to low copy numbers or high probe set background. This might be important when interpreting data from microarrays (Georgantas, III et al., 2004).

Ng *et al.* also investigated gene expression profilies of $CD34^+$ HSCs cells from bone marrow, UCB and mobilised PBSCs (Ng et al., 2004). They highlighted the importance in understanding the differences between bone marrow derived cells to UCB and peripheral blood derived cells, as these cells are being used theurapeutically. They found 51 genes between bone marrow and UCB to be expressed differentially and 64 genes between bone marrow and PBSCs (Ng et al., 2004).

To investigate proliferation and differentiation of HSCs, Venezia *et al.* looked at HSCs that were induced to proliferate and then return to quiescence *in vivo* (Venezia et al., 2004). This microarray data was then compared to data on naturally proliferating foetal HSCs and quiescent adult counterparts. It was found that the majority of quiescent HSCs expressed receptors (e.g. *IGF1 receptor* and *Tie1*) that allowed them to respond to multiple mitogenic signals. They also expressed high levels of transcription factors (e.g. *c-fos, GATA2*) which allowed them to be quickly activated. After activation the cells entered a superquiescent 'pause' mediated by anti-proliferative genes such as *Tob1, p21, Btg3* and interferon gamma induced genes (suggesting HSCs respond to pro-inflammatory signals). Then early proliferation markers (*Ki67, α4 integrin*) were expressed followed by late proliferation markers (*Cyclin B2, Sca1, CD48*) and there was a reset of quiescence (*Btg1, SOCS3 and endoglin*).

In liver, genes specific for rat oval cell have been investigated by Batusic *et al.* using microarray analysis (Batusic et al., 2005). Oval cells were generated in the rat 2-AAF/PH model and 12 genes were identified to be enriched oval cells (compared to AAF treatment and PH alone). Furthermore three genes (*thymidine kinase 1, Jun-D* and *ADP-ribosylation factor 4*) were upregulated in both oval cell dependent and normal liver regeneration by PH (but not by acute phase reaction). *Rab-3b* and *Ear-2*, on the other hand were two specifically expressed genes in the oval cells (Batusic et al., 2005).

Petkov *et al.* used microarray patterns of rat liver development by studying embryonic differentiation *in vivo* from day 13 to adulthood (Petkov et al., 2004). Genes involved in development, morphogenesis, differentiation (signal transducers, cell adhesion, migration and matrix proteins), inflammation, blood coagulation, detoxification, serum proteins, amino acids, lipids and carbohydrate metabolism were regulated. Twenty-eight genes also were found to be overexpressed in foetal liver (not detected in adult liver) and could be used as potential markers for identification of liver progenitor cells (Petkov et al., 2004).

The same rat model was used for SAGE analysis to observe liver regeneration. The analysis showed 27 up-regulated and 18 down-regulated genes (Cimica et al., 2005). These genes were further analysed using real-time PCR. Eleven kinetically regulated genes were identified, 8 were induced early (1-3 days after PH) during regeneration [e.g. *Cyclin D1, Stathmin 1, Cd151*] and 2 together with AFP [*E-tropomodulin, Cdc42*] 7 days after PH (Cimica et al., 2005).

Even though microarray and SAGE data has not been able to provide a stem cell signature common between species or different stem cell types (adult and embryonic) it is opening up a new way of analysing and understanding stem cells. In the near future a more concise and comprehensive definition of a stem cell will hopefully emerge.

7.3.2 Stem cells, cancer and stem cell cancer

Another very interesting area in stem cell biology has been identified by interdisciplinary research with cancer biology. Many stem cell markers have been associated with different cancers and they have several common characteristics.

In earlier chapters many cancer-linked markers have been discussed, for example telomerase (*Chapter 4*) and ABCG2 (*Chapter 5*). Moreover, Wnt signalling has been implicated with stem cell control and its dysregulation with cancer (Reya and Clevers, 2005). Wnt signalling has been shown to be involved in crypt physiology and colon cancer, epidermal development and cancer, as well as, HSCs and leukaemias (Reya and Clevers, 2005).

In HSCs un-regulated stem cell growth is a likely scenario for at least acute myelogenous leukaemia and chronic myelogenous leukaemia (Attar and Scadden, 2004). Different parts of HSCs biology have been dissected by Attar *et al.* to show common regulatory elements between cancer and stem cells. These include cyclin-dependent kinase inhibitors, telomerase, homeobox genes, transcriptional repressors, transcriptional co-activators, cytokine growth factors Hedgehog, patched, smoothened and BMP, Notch and G-protein coupled receptors (Attar and Scadden, 2004).

In liver, Theise *et al.* has identified four cases of small tumours in livers with chronic hepatitis (Theise et al., 2003). All these samples were found to contain undifferentiated cells with morphological and immunohistochemical features that would be expected of hepatic progenitor cells. The cells merged with hepatocellular carcinoma, cholangiocarcinoma as well as mature hepatocytes within the tumour. Undifferentiated liver cells have also been identified in nodular hyperplasias (Roskams et al., 1996) and adenomas (Libbrecht et al., 2001).

It remains to be answered what parallels may be drawn between the two fields and if stem cell cancers are a fundamental mechanism for all cancers (Attar and Scadden, 2004).

7.4 Proposed research plan

If the work carried out to isolate, characterise and culture putative human liver progenitor cells were to be continued, I propose the following lines of research:

1) Cell sorting of different populations of cells using stem cell associated surface markers and analysing these by microarray (or similar method) to identify the most probable liver stem cell population. Only 1μg of RNA is now required (around $5x10^4$ cells from our non-parenchymal cell cultures). It is essential to get a vast array of markers as no definitive markers have been identified and a 'stem cell signature' is required. The markers need to be surface markers so that they can be used for pulling out the cells. Other methods of selection (e.g. attachment, dye efflux) might also be used. The 'stemness' of the selected cell populations could be validated using animal models with liver injury.

2) To investigate possible links with liver carcinomas and liver stem cells. Chemotherapeutic agents used to kill off cancer cells, but which might be tolerated by cancer stem cells could be very useful methods of selecting liver stem/progenitor cells.

Bibliography

Abeyta,M.J., Clark,A.T., Rodriguez,R.T., Bodnar,M.S., Pera,R.A., and Firpo,M.T. (2004). Unique gene expression signatures of independently-derived human embryonic stem cell lines. Hum. Mol. Genet. *13*, 601-608.

Akin,C. and Metcalfe,D.D. (2004). The biology of Kit in disease and the application of pharmacogenetics. J. Allergy Clin. Immunol. *114*, 13-19.

Alberts,B., Bray,D., Lewis,J., Raff,M., Roberts,K., and Watson,J.D. (1994). Cancer. In Molecular Biology of the Cell, B.Alberts, D.Bray, J.Lewis, M.Raff, K.Roberts, and J.D.Watson, eds. (New York: Garland Publishing Inc.), pp. 1255-1294.

Alison,M. (1998). Liver stem cells: a two compartment system. Curr. Opin. Cell Biol. *10*, 710-715.

Alison,M., Golding,M., Lalani,E.N., Nagy,P., Thorgeirsson,S., and Sarraf,C. (1997). Wholesale hepatocytic differentiation in the rat from ductular oval cells, the progeny of biliary stem cells. J Hepatol *26*, 343-352.

Alison,M., Golding,M., Lalani,e., and Sarraf,C. (1998b). Wound healing in the liver with particular reference to stem cells. Philos. Trans. R. Soc. Lond B Biol. Sci. *353*, 877-894.

Alison,M., Golding,M., Lalani,e., and Sarraf,C. (1998a). Wound healing in the liver with particular reference to stem cells. Philos Trans R Soc Lond B Biol Sci *353*, 877-894.

Alison,M., Poulsom,R., Jeffery,R., Dhillon,A., Quaglia,A., Jacob,J., Novelli,M., Prentice,G., Williamson,J., and Wright,N. (2000). Hepatocytes from non-hepatic adult stem cells. Nature *406*, 257.

Alison,M. and Sarraf,C. (1998). Hepatic stem cells. J. Hepatol. *29*, 676-682.

Alison,M.R. (2003). Tissue-based stem cells: ABC transporter proteins take centre stage. J. Pathol. *200*, 547-550.

Alison,M.R., Poulsom,R., and Forbes,S.J. (2001). Update on hepatic stem cells. Liver *21*, 367-373.

Alison,M.R., Poulsom,R., Otto,W.R., Vig,P., Brittan,M., Direkze,N.C., Lovell,M., Fang,T.C., Preston,S.L., and Wright,N.A. (2004a). Recipes for adult stem cell plasticity: fusion cuisine or readymade? J. Clin. Pathol. *57*, 113-120.

Alison,M.R., Vig,P., Russo,F., Bigger,B.W., Amofah,E., Themis,M., and Forbes,S. (2004b). Hepatic stem cells: from inside and outside the liver? Cell Prolif. *37*, 1-21.

Allain,J.E., Dagher,I., Mahieu-Caputo,D., LOUX,N., Andreoletti,M., Westerman,K., Briand,P., FRANCO,D., Leboulch,P., and Weber,A. (2002). Immortalization of a primate bipotent epithelial liver stem cell. Proc. Natl. Acad. Sci. U. S. A *99*, 3639-3644.

Antonchuk,J., Sauvageau,G., and Humphries,R.K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell *109*, 39-45.

Asakura,A. and Rudnicki,M.A. (2002). Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. Exp. Hematol. *30*, 1339-1345.

Attar,E.C. and Scadden,D.T. (2004). Regulation of hematopoietic stem cell growth. Leukemia *18*, 1760-1768.

Avital,I., Inderbitzin,D., Aoki,T., Tyan,D.B., Cohen,A.H., Ferraresso,C., Rozga,J., Arnaout,W.S., and Demetriou,A.A. (2001). Isolation, characterization, and transplantation of bone marrow-derived hepatocyte stem cells. Biochem Biophys Res Commun *288*, 156-164.

Azuma,H., Hirose,T., Fujii,H., Oe,S., Yasuchika,K., Fujikawa,T., and Yamaoka,Y. (2003). Enrichment of hepatic progenitor cells from adult mouse liver. Hepatology *37*, 1385-1394.

Baal,N., Reisinger,K., Jahr,H., Bohle,R.M., Liang,O., Munstedt,K., Rao,C.V., Preissner,K.T., and Zygmunt,M.T. (2004). Expression of transcription factor Oct-4 and other embryonic genes in CD133 positive cells from human umbilical cord blood. Thromb. Haemost. *92*, 767-775.

Banfi,A., Bianchi,G., Notaro,R., Luzzatto,L., Cancedda,R., and Quarto,R. (2002). Replicative Aging and Gene Expression in Long-Term Cultures of Human Bone Marrow Stromal Cells. Tissue Eng *8*, 901-910.

Batusic,D.S., Cimica,V., Chen,Y., Tron,K., Hollemann,T., Pieler,T., and Ramadori,G. (2005). Identification of genes specific to "oval cells" in the rat 2-acetylaminofluorene/partial hepatectomy model. Histochem. Cell Biol. 1-16.

Baumann,U., Crosby,H.A., Ramani,P., Kelly,D.A., and Strain,A.J. (1999). Expression of the stem cell factor receptor c-kit in normal and diseased pediatric liver: identification of a human hepatic progenitor cell? Hepatology *30*, 112-117.

Beck,W.T., Cirtain,M.C., Glover,C.J., Felsted,R.L., and Safa,A.R. (1988). Effects of indole alkaloids on multidrug resistance and labeling of P-glycoprotein by a photoaffinity analog of vinblastine. Biochem. Biophys. Res. Commun. *153*, 959-966.

Bhatia,M., Wang,J.C., Kapp,U., Bonnet,D., and Dick,J.E. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc. Natl. Acad. Sci. U. S. A *94*, 5320-5325.

BioTeach.
http://www.bioteach.ubc.ca/MolecularBiology/PainlessGeneExpressionProfiling/. 2005.

Blau,H.M., Brazelton,T.R., and Weimann,J.M. (2001). The evolving concept of a stem cell: entity or function? Cell *105*, 829-841.

Block,G.D., Locker,J., Bowen,W.C., Petersen,B.E., Katayal,S., Strom,S.C., Riley,T., Howard,T.A., and Michalopoulos,G.K. (1996). Population expansion, clonal growth, and specific differentiation patterns in primary cultures of hepatocytes induced by

HGF/SF, EGF and TGF alpha in a chemically defined (HGM) medium. J. Cell Biol. *132*, 1133-1149.

Bodnar,A.G., Ouellette,M., Frolkis,M., Holt,S.E., Chiu,C.P., Morin,G.B., Harley,C.B., Shay,J.W., Lichtsteiner,S., and Wright,W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. Science *279*, 349-352.

Brill,S., Holst,P., Sigal,S., Zvibel,I., Fiorino,A., Ochs,A., Somasundaran,U., and Reid,L.M. (1993). Hepatic progenitor populations in embryonic, neonatal, and adult liver. Proc Soc Exp Biol Med *204*, 261-269.

Broudy,V.C., Lin,N., Zsebo,K.M., Birkett,N.C., Smith,K.A., Bernstein,I.D., and Papayannopoulou,T. (1992). Isolation and characterization of a monoclonal antibody that recognizes the human c-kit receptor. Blood *79*, 338-346.

Bunting,K.D. (2002). ABC transporters as phenotypic markers and functional regulators of stem cells. Stem Cells *20*, 11-20.

Bunting,K.D. and Hawley,R.G. (2003). Integrative molecular and developmental biology of adult stem cells. Biol. Cell *95*, 563-578.

Camargo,F.D., Finegold,M., and Goodell,M.A. (2004). Hematopoietic myelomonocytic cells are the major source of hepatocyte fusion partners. J. Clin. Invest *113*, 1266-1270.

Cann, A. Retroviral Vectors. 1998.
http://www.tulane.edu/~dmsander/WWW/335/peel/peel2.html

Cantz,T., Zuckerman,D.M., Burda,M.R., Dandri,M., Goricke,B., Thalhammer,S., Heckl,W.M., Manns,M.P., Petersen,J., and Ott,M. (2003). Quantitative gene expression analysis reveals transition of fetal liver progenitor cells to mature hepatocytes after transplantation in uPA/RAG-2 mice. Am. J. Pathol. *162*, 37-45.

Carpenter,M.K., Rosler,E., and Rao,M.S. (2003). Characterization and differentiation of human embryonic stem cells. Cloning Stem Cells *5*, 79-88.

Chambers,I., Colby,D., Robertson,M., Nichols,J., Lee,S., Tweedie,S., and Smith,A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643-655.

Chaudhary,P.M. and Roninson,I.B. (1991). Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell *66*, 85-94.

Chinzei,R., Tanaka,Y., Shimizu-Saito,K., Hara,Y., Kakinuma,S., Watanabe,M., Teramoto,K., Arii,S., Takase,K., Sato,C., Terada,N., and Teraoka,H. (2002). Embryoid-body cells derived from a mouse embryonic stem cell line show differentiation into functional hepatocytes. Hepatology *36*, 22-29.

Chiu,C.P., Dragowska,W., Kim,N.W., Vaziri,H., Yui,J., Thomas,T.E., Harley,C.B., and Lansdorp,P.M. (1996). Differential expression of telomerase activity in hematopoietic progenitors from adult human bone marrow. Stem Cells *14*, 239-248.

Cimica,V., Batusic,D., Chen,Y., Hollemann,T., Pieler,T., and Ramadori,G. (2005). Transcriptome analysis of rat liver regeneration in a model of oval hepatic stem cells. Genomics *86*, 352-364.

Coleman,W.B., Wennerberg,A.E., Smith,G.J., and Grisham,J.W. (1993). Regulation of the differentiation of diploid and some aneuploid rat liver epithelial (stemlike) cells by the hepatic microenvironment. Am J Pathol *142*, 1373-1382.

Counter,C.M., Hahn,W.C., Wei,W., Caddle,S.D., Beijersbergen,R.L., Lansdorp,P.M., Sedivy,J.M., and Weinberg,R.A. (1998a). Dissociation among in vitro telomerase activity, telomere maintenance, and cellular immortalization. Proc Natl Acad Sci U S A *95*, 14723-14728.

Counter,C.M., Meyerson,M., Eaton,E.N., Ellisen,L.W., Caddle,S.D., Haber,D.A., and Weinberg,R.A. (1998b). Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. Oncogene. *16*, 1217-1222.

Couvelard,A., Bringuier,A.F., Dauge,M.C., Nejjari,M., Darai,E., Benifla,J.L., Feldmann,G., Henin,D., and Scoazek,J.Y. (1998). Expression of integrins during liver organogenesis in humans. Hepatology *27*, 839-847.

Craig,C.E., Quaglia,A., and Dhillon,A.P. (2004a). Extramedullary haematopoiesis in massive hepatic necrosis. Histopathology *45*, 518-525.

Craig,C.E., Quaglia,A., Selden,C., Lowdell,M., Hodgson,H., and Dhillon,A.P. (2004b). The histopathology of regeneration in massive hepatic necrosis. Semin. Liver Dis. *24*, 49-64.

Crosby,H.A., Hubscher,S., Fabris,L., Joplin,R., Sell,S., Kelly,D., and Strain,A.J. (1998). Immunolocalization of putative human liver progenitor cells in livers from patients with end-stage primary biliary cirrhosis and sclerosing cholangitis using the monoclonal antibody OV-6. Am J Pathol *152*, 771-779.

Crosby,H.A., Kelly,D.A., and Strain,A.J. (2001). Human Hepatic Stem-like Cells Isolated Using c-kit or CD34 Can Differentiate Into Biliary Epithelium. Gastroenterology *120*, 534-544.

Crosby,H.A., Nijjar,S.S., de,G.J., V, Kelly,D.A., and Strain,A.J. (2002). Progenitor cells of the biliary epithelial cell lineage. Semin. Cell Dev. Biol. *13*, 397-403.

Dabeva,M.D., Petkov,P.M., Sandhu,J., Oren,R., Laconi,E., Hurston,E., and Shafritz,D.A. (2000). Proliferation and differentiation of fetal liver epithelial progenitor cells after transplantation into adult rat liver. Am J Pathol *156*, 2017-2031.

Dahlke,M.H., Popp,F.C., Larsen,S., Schlitt,H.J., and Rasko,J.E. (2004). Stem cell therapy of the liver--fusion or fiction? Liver Transpl. *10*, 471-479.

Dahlke,M.H. and Schlitt,H.J. (2003). Making hepatocytes from stem cells: where are we? Liver Transpl. *9*, 1100-1101.

Danet,G.H., Luongo,J.L., Butler,G., Lu,M.M., Tenner,A.J., Simon,M.C., and Bonnet,D.A. (2002). C1qRp defines a new human stem cell population with hematopoietic and hepatic potential. Proc. Natl. Acad. Sci. U. S. A *99*, 10441-10445.

De Vos,R. and Desmet,V. (1992). Ultrastructural characteristics of novel epithelial cell types identified in human pathologic liver specimens with chronic ductular reaction. Am. J. Pathol. *140*, 1441-1450.

de Wynter,E.A., Buck,D., Hart,C., Heywood,R., Coutinho,L.H., Clayton,A., Rafferty,J.A., Burt,D., Guenechea,G., Bueren,J.A., Gagen,D., Fairbairn,L.J., Lord,B.I., and Testa,N.G. (1998). CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors. Stem Cells *16*, 387-396.

de Wynter,E.A., Coutinho,L.H., Pei,X., Marsh,J.C., Hows,J., Luft,T., and Testa,N.G. (1995). Comparison of purity and enrichment of CD34+ cells from bone marrow, umbilical cord and peripheral blood (primed for apheresis) using five separation systems. Stem Cells *13*, 524-532.

Deng,J., Steindler,D.A., Laywell,E.D., and Petersen,B.E. (2003). Neural trans-differentiation potential of hepatic oval cells in the neonatal mouse brain. Exp. Neurol. *182*, 373-382.

Devine,S.M., Cobbs,C., Jennings,M., Bartholomew,A., and Hoffman,R. (2003). Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood *101*, 2999-3001.

Dhaene,K., Van Marck,E., and Parwaresch,R. (2000). Telomeres, telomerase and cancer: an up-date. Virchows Arch. *437*, 1-16.

Di Campli,C., Piscaglia,A.C., Pierelli,L., Rutella,S., Bonanno,G., Alison,M.R., Mariotti,A., Vecchio,F.M., Nestola,M., Monego,G., Michetti,F., Mancuso,S., Pola,P., Leone,G., Gasbarrini,G., and Gasbarrini,A. (2004). A human umbilical cord stem cell rescue therapy in a murine model of toxic liver injury. Dig. Liver Dis. *36*, 603-613.

Dimmock,N.J. and Primrose,S.B. (1994a). Carcinogenesis and tumour viruses. In Introduction to Modern Virology, N.J.Dimmock and S.B.Primrose, eds. (Oxford: Blackwell Science), pp. 256-275.

Dimmock,N.J. and Primrose,S.B. (1994b). The process of infection: IID. RNA viruses with a DNA intermediate and vice versa. In Introduction to Modern Virology, N.J.Dimmock and S.B.Primrose, eds. (Oxford: Blackwell Science), pp. 133-142.

Dimri,G.P., Lee,X., Basile,G., Acosta,M., Scott,G., Roskelley,C., Medrano,E.E., Linskens,M., Rubelj,I., Pereira-Smith,O., and . (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. U. S. A *92*, 9363-9367.

Domen,J. and Weissman,I.L. (1999). Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. Mol. Med. Today *5*, 201-208.

Doyle,L.A. and Ross,D.D. (2003). Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). Oncogene *22*, 7340-7358.

Draper,J.S., Pigott,C., Thomson,J.A., and Andrews,P.W. (2002). Surface antigens of human embryonic stem cells: changes upon differentiation in culture. J. Anat. *200*, 249-258.

Ejendal,K.F. and Hrycyna,C.A. (2002). Multidrug resistance and cancer: the role of the human ABC transporter ABCG2. Curr. Protein Pept. Sci. *3*, 503-511.

Elwood,N.J., Jiang,X.R., Chiu,C.P., Lebkowski,J.S., and Smith,C.A. (2004). Enhanced long-term survival, but no increase in replicative capacity, following retroviral transduction of human cord blood CD34+ cells with human telomerase reverse transcriptase. Haematologica *89*, 377-378.

Engelhardt,M., Kumar,R., Albanell,J., Pettengell,R., Han,W., and Moore,M.A. (1997). Telomerase regulation, cell cycle, and telomere stability in primitive hematopoietic cells. Blood *90*, 182-193.

Evarts,R.P., Hu,Z., Omori,N., Omori,M., Marsden,E.R., and Thorgeirsson,S.S. (1996). Precursor-product relationship between oval cells and hepatocytes: comparison between tritiated thymidine and bromodeoxyuridine as tracers. Carcinogenesis. *17*, 2143-2151.

Fang,T.C., Alison,M.R., Wright,N.A., and Poulsom,R. (2004). Adult stem cell plasticity: will engineered tissues be rejected? Int. J. Exp. Pathol. *85*, 115-124.

Faris,R.A., Konkin,T., and Halpert,G. (2001). Liver stem cells: a potential source of hepatocytes for the treatment of human liver disease. Artif. Organs *25*, 513-521.

Fausto,N. (2005). Tweaking liver progenitor cells. Nat. Med. *11*, 1053-1054.

Fausto,N., Laird,A.D., and Webber,E.M. (1995). Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. FASEB J. *9*, 1527-1536.

Fiegel,H.C., Lioznov,M.V., Cortes-Dericks,L., Lange,C., Kluth,D., Fehse,B., and Zander,A.R. (2003a). Liver-specific gene expression in cultured human hematopoietic stem cells. Stem Cells *21*, 98-104.

Fiegel,H.C., Park,J.J., Lioznov,M.V., Martin,A., Jaeschke-Melli,S., Kaufmann,P.M., Fehse,B., Zander,A.R., and Kluth,D. (2003b). Characterization of cell types during rat liver development. Hepatology *37*, 148-154.

Forbes,S., Vig,P., Poulsom,R., Thomas,H., and Alison,M. (2002). Hepatic stem cells. J. Pathol. *197*, 510-518.

Fortunel,N.O., Otu,H.H., Ng,H.H., Chen,J., Mu,X., Chevassut,T., Li,X., Joseph,M., Bailey,C., Hatzfeld,J.A., Hatzfeld,A., Usta,F., Vega,V.B., Long,P.M., Libermann,T.A., and Lim,B. (2003). Comment on " 'Stemness': transcriptional profiling of embryonic and adult stem cells" and "a stem cell molecular signature". Science *302*, 393.

Fuchs,E. and Segre,J.A. (2000). Stem cells: a new lease on life. Cell *100*, 143-155.

Fujikawa,T., Hirose,T., Fujii,H., Oe,S., Yasuchika,K., Azuma,H., and Yamaoka,Y. (2003). Purification of adult hepatic progenitor cells using green fluorescent protein (GFP)-transgenic mice and fluorescence-activated cell sorting. J. Hepatol. *39*, 162-170.

Fujio,K., Hu,Z., Evarts,R.P., Marsden,E.R., NIU,C.H., and Thorgeirsson,S.S. (1996). Coexpression of stem cell factor and c-kit in embryonic and adult liver. Exp. Cell Res. *224*, 243-250.

Gallacher,L., Murdoch,B., Wu,D.M., Karanu,F.N., Keeney,M., and Bhatia,M. (2000). Isolation and characterization of human CD34(-)Lin(-) and CD34(+)Lin(-) hematopoietic stem cells using cell surface markers AC133 and CD7. Blood *95*, 2813-2820.

Georgantas,R.W., III, Tanadve,V., Malehorn,M., Heimfeld,S., Chen,C., Carr,L., Martinez-Murillo,F., Riggins,G., Kowalski,J., and Civin,C.I. (2004). Microarray and serial analysis of gene expression analyses identify known and novel transcripts overexpressed in hematopoietic stem cells. Cancer Res. *64*, 4434-4441.

Goff,J.P., Shields,D.S., Petersen,B.E., Zajac,V.F., Michalopoulos,G.K., and Greenberger,J.S. (1996). Synergistic effects of hepatocyte growth factor on human cord blood CD34+ progenitor cells are the result of c-met receptor expression. Stem. Cells *14*, 592-602.

Gomez-Lechon,M.J. (1999). Oncostatin M: signal transduction and biological activity. Life Sci. *65*, 2019-2030.

Goodell,M.A., Brose,K., Paradis,G., Conner,A.S., and Mulligan,R.C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J. Exp. Med. *183*, 1797-1806.

Goodell,M.A., Rosenzweig,M., Kim,H., Marks,D.F., DeMaria,M., Paradis,G., Grupp,S.A., Sieff,C.A., Mulligan,R.C., and Johnson,R.P. (1997). Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat. Med *3*, 1337-1345.

Gordon,G.J., Butz,G.M., Grisham,J.W., and Coleman,W.B. (2002). Isolation, short-term culture, and transplantation of small hepatocyte-like progenitor cells from retrorsine-exposed rats. Transplantation *73*, 1236-1243.

Gordon,G.J., Coleman,W.B., and Grisham,J.W. (2000a). Temporal analysis of hepatocyte differentiation by small hepatocyte-like progenitor cells during liver regeneration in retrorsine-exposed rats. Am J Pathol *157*, 771-786.

Gordon,G.J., Coleman,W.B., Hixson,D.C., and Grisham,J.W. (2000b). Liver regeneration in rats with retrorsine-induced hepatocellular injury proceeds through a novel cellular response. Am. J. Pathol. *156*, 607-619.

Gordon,P.R., Leimig,T., Babarin-Dorner,A., Houston,J., Holladay,M., Mueller,I., Geiger,T., and Handgretinger,R. (2003). Large-scale isolation of CD133+ progenitor cells from G-CSF mobilized peripheral blood stem cells. Bone Marrow Transplant. *31*, 17-22.

Gupta,P., Oegema,T.R., Jr., Brazil,J.J., Dudek,A.Z., Slungaard,A., and Verfaillie,C.M. (2000). Human LTC-IC can be maintained for at least 5 weeks in vitro when interleukin-3 and a single chemokine are combined with O-sulfated heparan sulfates: requirement for optimal binding interactions of heparan sulfate with early-acting cytokines and matrix proteins. Blood *95*, 147-155.

Hamazaki,T., Iiboshi,Y., Oka,M., Papst,P.J., Meacham,A.M., Zon,L.I., and Terada,N. (2001). Hepatic maturation in differentiating embryonic stem cells in vitro. FEBS. Lett *497*, 15-19.

Hannon,G.J., Sun,P., Carnero,A., Xie,L.Y., Maestro,R., Conklin,D.S., and Beach,D. (1999). MaRX: an approach to genetics in mammalian cells. Science *283*, 1129-1130.

Harle-Bachor,C. and Boukamp,P. (1996). Telomerase activity in the regenerative basal layer of the epidermis inhuman skin and in immortal and carcinoma-derived skin keratinocytes. Proc. Natl. Acad. Sci. U. S. A *93*, 6476-6481.

Harrington,L. (2004). Does the reservoir for self-renewal stem from the ends? Oncogene *%20;23*, 7283-7289.

Hatch,H.M., Zheng,D., Jorgensen,M.L., and Petersen,B.E. (2002). SDF-1alpha/CXCR4: a mechanism for hepatic oval cell activation and bone marrow stem cell recruitment to the injured liver of rats. Cloning Stem Cells *4*, 339-351.

He,Z.P., Tan,W.Q., Tang,Y.F., and Feng,M.F. (2003). Differentiation of putative hepatic stem cells derived from adult rats into mature hepatocytes in the presence of epidermal growth factor and hepatocyte growth factor. Differentiation *71*, 281-290.

He,Z.P., Tan,W.Q., Tang,Y.F., Zhang,H.J., and Feng,M.F. (2004). Activation, isolation, identification and in vitro proliferation of oval cells from adult rat livers. Cell Prolif. *37*, 177-187.

Henderson,J.K., Draper,J.S., Baillie,H.S., Fishel,S., Thomson,J.A., Moore,H., and Andrews,P.W. (2002). Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells *20*, 329-337.

Herzog,E.L., Chai,L., and Krause,D.S. (2003). Plasticity of marrow-derived stem cells. Blood *102*, 3483-3493.

Hollo,Z., Homolya,L., Davis,C.W., and Sarkadi,B. (1994). Calcein accumulation as a fluorometric functional assay of the multidrug transporter. Biochim. Biophys. Acta *1191*, 384-388.

Homolya,L., Hollo,Z., Germann,U.A., Pastan,I., Gottesman,M.M., and Sarkadi,B. (1993). Fluorescent cellular indicators are extruded by the multidrug resistance protein. J. Biol. Chem. *268*, 21493-21496.

Hoppo,T., Fujii,H., Hirose,T., Yasuchika,K., Azuma,H., Baba,S., Naito,M., Machimoto,T., and Ikai,I. (2004). Thy1-positive mesenchymal cells promote the maturation of CD49f-positive hepatic progenitor cells in the mouse fetal liver. Hepatology *39*, 1362-1370.

Hove,W.R., van Hoek,B., Bajema,I.M., Ringers,J., van Krieken,J.H., and Lagaaij,E.L. (2003). Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. Liver Transpl. *9*, 552-556.

Hu,A., Cai,J., Zheng,Q., He,X., Pan,Y., and Li,L. (2003). Hepatic differentiation from embryonic stem cells in vitro. Chin Med. J. (Engl.) *116*, 1893-1897.

Imai,Y., Nakane,M., Kage,K., Tsukahara,S., Ishikawa,E., Tsuruo,T., Miki,Y., and Sugimoto,Y. (2002). C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance. Mol. Cancer Ther. *1*, 611-616.

Ishizaka,S., Shiroi,A., Kanda,S., Yoshikawa,M., Tsujinoue,H., Kuriyama,S., Hasuma,T., Nakatani,K., and Takahashi,K. (2002). Development of hepatocytes from ES cells after transfection with the HNF-3beta gene. FASEB J. *16*, 1444-1446.

Ivanova,N.B., Dimos,J.T., Schaniel,C., Hackney,J.A., Moore,K.A., and Lemischka,I.R. (2002). A stem cell molecular signature. Science *298*, 601-604.

Jakubowski,A., Ambrose,C., Parr,M., Lincecum,J.M., Wang,M.Z., Zheng,T.S., Browning,B., Michaelson,J.S., Baestcher,M., Wang,B., Bissell,D.M., and Burkly,L.C. (2005). TWEAK induces liver progenitor cell proliferation. J. Clin. Invest *115*, 2330-2340.

Jang,Y.Y., Collector,M.I., Baylin,S.B., Diehl,A.M., and Sharkis,S.J. (2004). Hematopoietic stem cells convert into liver cells within days without fusion. Nat. Cell Biol. *6*, 532-539.

Jiang,Y., Jahagirdar,B.N., Reinhardt,R.L., Schwartz,R.E., Keene,C.D., Ortiz-Gonzalez,X.R., Reyes,M., Lenvik,T., Lund,T., Blackstad,M., Du,J., Aldrich,S., Lisberg,A., Low,W.C., Largaespada,D.A., and Verfaillie,C.M. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. Nature *418*, 41-49.

Joplin,R., Hishida,T., Tsubouchi,H., Daikuhara,Y., Ayres,R., Neuberger,J.M., and Strain,A.J. (1992). Human intrahepatic biliary epithelial cells proliferate in vitro in response to human hepatocyte growth factor. J. Clin. Invest. *90*, 1284-1289.

Joplin,R., Strain,A.J., and Neuberger,J.M. (1989). Immuno-isolation and culture of biliary epithelial cells from normal human liver. In Vitro Cell Dev. Biol. *25*, 1189-1192.

Joplin,R., Strain,A.J., and Neuberger,J.M. (1990). Biliary epithelial cells from the liver of patients with primary biliary cirrhosis: isolation, characterization, and short-term culture. J. Pathol. *162*, 255-260.

Kakinuma,S., Tanaka,Y., Chinzei,R., Watanabe,M., Shimizu-Saito,K., Hara,Y., Teramoto,K., Arii,S., Sato,C., Takase,K., Yasumizu,T., and Teraoka,H. (2003). Human umbilical cord blood as a source of transplantable hepatic progenitor cells. Stem Cells *21*, 217-227.

Kamiya,A., Kinoshita,T., Ito,Y., Matsui,T., Morikawa,Y., Senba,E., Nakashima,K., Taga,T., Yoshida,K., Kishimoto,T., and Miyajima,A. (1999). Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. EMBO J. *18*, 2127-2136.

Kamiya,A., Kinoshita,T., and Miyajima,A. (2001). Oncostatin M and hepatocyte growth factor induce hepatic maturation via distinct signaling pathways. FEBS. Lett *492*, 90-94.

Kano,J., Noguchi,M., Kodama,M., and Tokiwa,T. (2000). The in vitro differentiating capacity of nonparenchymal epithelial cells derived from adult porcine livers. Am. J. Pathol. *156*, 2033-2043.

Katoh,M., Katoh,M., Kameyama,M., Kugoh,H., Shimizu,M., and Oshimura,M. (1998). A repressor function for telomerase activity in telomerase-negative immortal cells. Mol. Carcinog. *21*, 17-25.

Kilian,A., Bowtell,D.D., Abud,H.E., Hime,G.R., Venter,D.J., Keese,P.K., Duncan,E.L., Reddel,R.R., and Jefferson,R.A. (1997). Isolation of a candidate human telomerase

catalytic subunit gene, which reveals complex splicing patterns in different cell types. Hum. Mol. Genet. *6*, 2011-2019.

Kim,M., Turnquist,H., Jackson,J., Sgagias,M., Yan,Y., Gong,M., Dean,M., Sharp,J.G., and Cowan,K. (2002). The multidrug resistance transporter ABCG2 (breast cancer resistance protein 1) effluxes Hoechst 33342 and is overexpressed in hematopoietic stem cells. Clin. Cancer Res. *8*, 22-28.

Kim,N.W., Piatyszek,M.A., Prowse,K.R., Harley,C.B., West,M.D., Ho,P.L., Coviello,G.M., Wright,W.E., Weinrich,S.L., and Shay,J.W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011-2015.

Kinoshita,T. and Miyajima,A. (2002). Cytokine regulation of liver development. Biochim. Biophys. Acta *1592*, 303-312.

Kinoshita,T., Sekiguchi,T., Xu,M.J., Ito,Y., Kamiya,A., Tsuji,K., Nakahata,T., and Miyajima,A. (1999). Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. Proc. Natl. Acad. Sci. U. S. A *96*, 7265-7270.

Kobari,L., Giarratana,M.C., Pflumio,F., Izac,B., Coulombel,L., and Douay,L. (2001). CD133+ cell selection is an alternative to CD34+ cell selection for ex vivo expansion of hematopoietic stem cells. J. Hematother. Stem Cell Res. *10*, 273-281.

Kollet,O., Shivtiel,S., Chen,Y.Q., Suriawinata,J., Thung,S.N., Dabeva,M.D., Kahn,J., Spiegel,A., Dar,A., Samira,S., Goichberg,P., Kalinkovich,A., Arenzana-Seisdedos,F., Nagler,A., Hardan,I., Revel,M., Shafritz,D.A., and Lapidot,T. (2003). HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. J. Clin. Invest *112*, 160-169.

Kolquist,K.A., Ellisen,L.W., Counter,C.M., Meyerson,M., Tan,L.K., Weinberg,R.A., Haber,D.A., and Gerald,W.L. (1998). Expression of TERT in early premalignant lesions and a subset of cells in normal tissues. Nat. Genet. *19*, 182-186.

Korbling,M., Katz,R.L., Khanna,A., Ruifrok,A.C., Rondon,G., Albitar,M., Champlin,R.E., and Estrov,Z. (2002). Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N. Engl. J. Med. *346*, 738-746.

Kountouras,J., Boura,P., and Lygidakis,N.J. (2001). Liver regeneration after hepatectomy. Hepatogastroenterology *48*, 556-562.

Kuai,X.L., Cong,X.Q., Li,X.L., and Xiao,S.D. (2003). Generation of hepatocytes from cultured mouse embryonic stem cells. Liver Transpl. *9*, 1094-1099.

Kuci,S., Wessels,J.T., Buhring,H.J., Schilbach,K., Schumm,M., Seitz,G., Loffler,J., Bader,P., Schlegel,P.G., Niethammer,D., and Handgretinger,R. (2003). Identification of a novel class of human adherent CD34- stem cells that give rise to SCID-repopulating cells. Blood *101*, 869-876.

Kucia,M., Ratajczak,J., Reca,R., Janowska-Wieczorek,A., and Ratajczak,M.Z. (2004). Tissue-specific muscle, neural and liver stem/progenitor cells reside in the bone marrow, respond to an SDF-1 gradient and are mobilized into peripheral blood during stress and tissue injury. Blood Cells Mol. Dis. *32*, 52-57.

Lagasse,E., Connors,H., Al Dhalimy,M., Reitsma,M., Dohse,M., Osborne,L., Wang,X., Finegold,M., Weissman,I.L., and Grompe,M. (2000). Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med *6*, 1229-1234.

Laurson, J., Clements, C. O., Mavri, D., Selden C, and Hodgson HJF. Cell colonies expressing both hepatocyte and biliary epithelial cell markers can be isolated from non-parenchymal cells of alcohol cirrhotic liver explants. Poster at BASL 2004 . 9-9-2004.

Laurson, J., Mavri, D., Oakley, P., Hodgson, H, and Selden, C. A proliferating population of human cells expressing hepatocyte and biliary epithelial cell markers cultured long term in non-differentiating conditions. Oral presentation at EASL monothematic conference Strategies for liver support: From Stem Cells to Xenotransplantation . 9-25-2003.

Lavon,N., Yanuka,O., and Benvenisty,N. (2004). Differentiation and isolation of hepatic-like cells from human embryonic stem cells. Differentiation *72*, 230-238.

Lazaro,C.A., Croager,E.J., Mitchell,C., Campbell,J.S., Yu,C., Foraker,J., Rhim,J.A., Yeoh,G.C., and Fausto,N. (2003). Establishment, characterization, and long-term maintenance of cultures of human fetal hepatocytes. Hepatology *38*, 1095-1106.

Libbrecht,L., De Vos,R., Cassiman,D., Desmet,V., Aerts,R., and Roskams,T. (2001). Hepatic progenitor cells in hepatocellular adenomas. Am. J. Surg. Pathol. *25*, 1388-1396.

Libbrecht,L., Desmet,V., Van Damme,B., and Roskams,T. (2000). Deep intralobular extension of human hepatic 'progenitor cells' correlates with parenchymal inflammation in chronic viral hepatitis: can 'progenitor cells' migrate? [In Process Citation]. J Pathol *192*, 373-378.

Linnekin,D., Keller,J.R., Ferris,D.K., Mou,S.M., Broudy,V., and Longo,D.L. (1995). Stem cell factor induces phosphorylation of a 200 kDa protein which associates with c-kit. Growth Factors *12*, 57-67.

Litman,T., Brangi,M., Hudson,E., Fetsch,P., Abati,A., Ross,D.D., Miyake,K., Resau,J.H., and Bates,S.E. (2000). The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J. Cell Sci. *113 (Pt 11)*, 2011-2021.

Lowdell,M. (2001). Data analysis in flow cytometry. In Cytometric analysis of cell phenotype and function, D.McCarthy and M.G.Macey, eds. (Cambridge UK: Cambridge University Press).

Lowdell, M. Flow Cytometry Course - Royal Free Hospital. 6-10-2003. Personal Communication

Lowes,K.N., Brennan,B.A., Yeoh,G.C., and Olynyk,J.K. (1999). Oval cell numbers in human chronic liver diseases are directly related to disease severity. Am J Pathol *154*, 537-541.

Machalinski,B., Wiszniewska,B., Baskiewicz,M., Marchlewicz,M., Majka,M., Wenda-Rozewicka,L., and Ratajczak,M.Z. (1998). In vivo and in vitro studies on the toxicity of

Hoechst 33342 (Ho342). Implications for employing Ho342 for the isolation of haematopoietic stem cells. Ann. Transplant. *3*, 5-13.

Malhi,H., Irani,A.N., Gagandeep,S., and Gupta,S. (2002). Isolation of human progenitor liver epithelial cells with extensive replication capacity and differentiation into mature hepatocytes. J. Cell Sci. *115*, 2679-2688.

Marek,C.J., Cameron,G.A., Elrick,L.J., Hawksworth,G.M., and Wright,M.C. (2003). Generation of hepatocytes expressing functional cytochromes P450 from a pancreatic progenitor cell line in vitro. Biochem. J. *370*, 763-769.

Masutomi,K., Yu,E.Y., Khurts,S., Ben Porath,I., Currier,J.L., Metz,G.B., Brooks,M.W., Kaneko,S., Murakami,S., DeCaprio,J.A., Weinberg,R.A., Stewart,S.A., and Hahn,W.C. (2003). Telomerase maintains telomere structure in normal human cells. Cell *114*, 241-253.

Matsumura,T., Takesue,M., Westerman,K.A., Okitsu,T., Sakaguchi,M., Fukazawa,T., Totsugawa,T., Noguchi,H., Yamamoto,S., Stolz,D.B., Tanaka,N., Leboulch,P., and Kobayashi,N. (2004). Establishment of an immortalized human-liver endothelial cell line with SV40T and hTERT. Transplantation *77*, 1357-1365.

McLaren,A. (2001). Ethical and social considerations of stem cell research. Nature. *414*, 129-131.

Medical Dictionary. Definition of Stem Cell. 2003. http://cancerweb.ncl.ac.uk/cgi-bin/omd?query=stem+cell

Mezey,E. (2004). Commentary: on bone marrow stem cells and openmindedness. Stem Cells Dev. *13*, 147-152.

Michalopoulos,G.K., Barua,L., and Bowen,W.C. (2005). Transdifferentiation of rat hepatocytes into biliary cells after bile duct ligation and toxic biliary injury. Hepatology *41*, 535-544.

Miki, T, Cai, H., and Strom, S. C. Production of hepatocytes from human amniotic stem cells. Hepatology 36[4Pt2], 171A. 2002.

Miki, T, Lehmann, T., Cai, H., and Strom, S. Isolation of multipotent stem cells from placenta. Hepatology - AASLD Abstracts 38[4], 290A. 2003.

Minguet,S., Cortegano,I., Gonzalo,P., Martinez-Marin,J.A., de Andres,B., Salas,C., Melero,D., Gaspar,M.L., and Marcos,M.A. (2003). A population of c-Kit(low)(CD45/TER119)- hepatic cell progenitors of 11-day postcoitus mouse embryo liver reconstitutes cell-depleted liver organoids. J. Clin. Invest *112*, 1152-1163.

Miraglia,S., Godfrey,W., Yin,A.H., Atkins,K., Warnke,R., Holden,J.T., Bray,R.A., Waller,E.K., and Buck,D.W. (1997). A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. Blood *90*, 5013-5021.

Mitaka,T., Mizuguchi,T., Sato,F., Mochizuki,C., and Mochizuki,Y. (1998). Growth and maturation of small hepatocytes. J. Gastroenterol. Hepatol. *13 Suppl*, S70-S77.

Mitsui,K., Tokuzawa,Y., ITOH,H., Segawa,K., Murakami,M., Takahashi,K., Maruyama,M., Maeda,M., and Yamanaka,S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell *113*, 631-642.

Miyazaki,M., Akiyama,I., Sakaguchi,M., Nakashima,E., Okada,M., Kataoka,K., and Huh,N.H. (2002). Improved conditions to induce hepatocytes from rat bone marrow cells in culture. Biochem. Biophys. Res. Commun. *298*, 24-30.

Mizuguchi,T., Hui,T., Palm,K., Sugiyama,N., Mitaka,T., Demetriou,A.A., and Rozga,J. (2001). Enhanced proliferation and differentiation of rat hepatocytes cultured with bone marrow stromal cells. J. Cell Physiol *189*, 106-119.

Molecular Probes. Molecular Probes web-site - Technical resources. 2005. http://probes.invitrogen.com/handbook/figures/0699.html

MUN. The Cell Cycle: DNA Replication, Mitosis and Cancer. 2005. http://www.mun.ca/biology/desmid/brian/BIOL2060/CellBiol17/CB17_14.html

Nakamura,K., Nonaka,H., SAITO,H., Tanaka,M., and Miyajima,A. (2004). Hepatocyte proliferation and tissue remodeling is impaired after liver injury in oncostatin M receptor knockout mice. Hepatology *39*, 635-644.

National Human Genome Research Institiute. http://www.genome.gov/10000533. 2005.

Nejjari,M., Couvelard,A., Mosnier,J.F., Moreau,A., Feldmann,G., Degott,C., Marcellin,P., and Scoazek,J.Y. (2001). Integrin up-regulation in chronic liver disease: relationship with inflammation and fibrosis in chronic hepatitis C. J. Pathol. *195*, 473-481.

Newsome,P.N., Johannessen,I., Boyle,S., Dalakas,E., McAulay,K.A., Samuel,K., Rae,F., Forrester,L., Turner,M.L., Hayes,P.C., Harrison,D.J., Bickmore,W.A., and Plevris,J.N. (2003). Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. Gastroenterology *124*, 1891-1900.

Ng,I.O., Chan,K.L., Shek,W.H., Lee,J.M., Fong,D.Y., Lo,C.M., and Fan,S.T. (2003). High frequency of chimerism in transplanted livers. Hepatology *38*, 989-998.

Ng,Y.Y., van Kessel,B., Lokhorst,H.M., Baert,M.R., van den Burg,C.M., Bloem,A.C., and Staal,F.J. (2004). Gene-expression profiling of CD34+ cells from various hematopoietic stem-cell sources reveals functional differences in stem-cell activity. J. Leukoc. Biol. *75*, 314-323.

Nichols,J., Zevnik,B., Anastassiadis,K., Niwa,H., Klewe-Nebenius,D., Chambers,I., Scholer,H., and Smith,A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379-391.

Nicoll, A. What is Fluorescent Activated Cell Sorting (FACS)? 2004.

Nitou,M., Sugiyama,Y., Ishikawa,K., and Shiojiri,N. (2002). Purification of fetal mouse hepatoblasts by magnetic beads coated with monoclonal anti-e-cadherin antibodies and their in vitro culture. Exp. Cell Res. *279*, 330-343.

Nonome,K., Li,X.K., Takahara,T., Kitazawa,Y., Funeshima,N., Yata,Y., Xue,F., Kanayama,M., Shinno,E., Kuwae,C., Saito,S., Watanabe,A., and Sugiyama,T. (2005). Human umbilical cord blood-derived cells differentiate into hepatocyte-like cells in the Fas-mediated liver injury model. Am. J. Physiol Gastrointest. Liver Physiol.

Nowak,G., Ericzon,B.G., Nava,S., Jaksch,M., Westgren,M., and Sumitran-Holgersson,S. (2005). Identification of expandable human hepatic progenitors which differentiate into mature hepatic cells in vivo. Gut *54*, 972-979.

Oben,J.A., Roskams,T., Yang,S., Lin,H., Sinelli,N., Li,Z., Torbenson,M., Huang,J., Guarino,P., Kafrouni,M., and Diehl,A.M. (2003). Sympathetic nervous system inhibition increases hepatic progenitors and reduces liver injury. Hepatology *38*, 664-673.

Oh,S.H., Hatch,H.M., and Petersen,B.E. (2002). Hepatic oval 'stem' cell in liver regeneration. Semin. Cell Dev. Biol. *13*, 405-409.

Oh,S.H., Miyazaki,M., Kouchi,H., Inoue,Y., Sakaguchi,M., Tsuji,T., Shima,N., Higashio,K., and Namba,M. (2000). Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro. Biochem. Biophys. Res. Commun. *279*, 500-504.

Okumoto,K., Saito,T., Hattori,E., Ito,J.I., Adachi,T., Takeda,T., Sugahara,K., Watanabe,H., Saito,K., Togashi,H., and Kawata,S. (2003). Differentiation of bone marrow cells into cells that express liver-specific genes in vitro: implication of the Notch signals in differentiation. Biochem. Biophys. Res. Commun. *304*, 691-695.

Olovnikov,A.M. (1973). A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. J. Theor. Biol. *41*, 181-190.

Omary,M.B., Ku,N.O., and Toivola,D.M. (2002). Keratins: guardians of the liver. Hepatology *35*, 251-257.

Omori,M., Omori,N., Evarts,R.P., Teramoto,T., and Thorgeirsson,S.S. (1997). Coexpression of flt-3 ligand/flt-3 and SCF/c-kit signal transduction system in bile-duct-ligated SI and W mice. Am J Pathol *150*, 1179-1187.

Ortolani, C. A multiparametric approach to immunophenotyping. Purdue Cytometry CD-ROM Series 3. 7-8-2004.

Overturf,K., Al Dhalimy,M., Tanguay,R., Brantly,M., Ou,C.N., Finegold,M., and Grompe,M. (1996). Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I [see comments] [published erratum appears in Nat Genet 1996 Apr;12(4):458]. Nat. Genet. *12*, 266-273.

Pan,Y.L., Cai,J.Y., and Hu,A.B. (2005). Differentiation of hepatocytes from mouse embryonic stem cells and its significance. Hepatobiliary. Pancreat. Dis. Int. *4*, 291-294.

Petersen,B.E. (2001). Hepatic "stem" cells: coming full circle. Blood Cells Mol Dis *27*, 590-600.

Petersen,B.E., Bowen,W.C., Patrene,K.D., Mars,W.M., Sullivan,A.K., Murase,N., Boggs,S.S., Greenberger,J.S., and Goff,J.P. (1999). Bone Marrow as a Potential Source of Hepatic Oval Cells. Science *284*, 1168-1170.

Petersen,B.E., Goff,J.P., Greenberger,J.S., and Michalopoulos,G.K. (1998). Hepatic oval cells express the hematopoietic stem cell marker Thy-1 in the rat. Hepatology *27*, 433-445.

Petersen,B.E., Grossbard,B., Hatch,H., Pi,L., Deng,J., and Scott,E.W. (2003). Mouse A6-positive hepatic oval cells also express several hematopoietic stem cell markers. Hepatology *37*, 632-640.

Petkov,P.M., Zavadil,J., Goetz,D., Chu,T., Carver,R., Rogler,C.E., Bottinger,E.P., Shafritz,D.A., and Dabeva,M.D. (2004). Gene expression pattern in hepatic stem/progenitor cells during rat fetal development using complementary DNA microarrays. Hepatology *39*, 617-627.

Pittenger,M.F., Mackay,A.M., Beck,S.C., Jaiswal,R.K., Douglas,R., Mosca,J.D., Moorman,M.A., Simonetti,D.W., Craig,S., and Marshak,D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.

Preston,S.L., Alison,M.R., Forbes,S.J., Direkze,N.C., Poulsom,R., and Wright,N.A. (2003). The new stem cell biology: something for everyone. Mol. Pathol. *56*, 86-96.

Qin,A.L., Zhou,X.Q., Zhang,W., Yu,H., and Xie,Q. (2004). Characterization and enrichment of hepatic progenitor cells in adult rat liver. World J. Gastroenterol. *10*, 1480-1486.

Ramalho-Santos,M., Yoon,S., Matsuzaki,Y., Mulligan,R.C., and Melton,D.A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. Science *298*, 597-600.

Rambhatla,L., Chiu,C.P., Kundu,P., Peng,Y., and Carpenter,M.K. (2003). Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant. *12*, 1-11.

Ramsfjell,V., Bryder,D., Bjorgvinsdottir,H., Kornfalt,S., Nilsson,L., Borge,O.J., and Jacobsen,S.E. (1999). Distinct requirements for optimal growth and In vitro expansion of human CD34(+)CD38(-) bone marrow long-term culture-initiating cells (LTC-IC), extended LTC-IC, and murine in vivo long-term reconstituting stem cells. Blood *94*, 4093-4102.

Rao,R.R. and Stice,S.L. (2004). Gene expression profiling of embryonic stem cells leads to greater understanding of pluripotency and early developmental events. Biol. Reprod. *71*, 1772-1778.

Ratajczak,J., Machalinski,B., Majka,M., Kijowski,J., Marlicz,W., Rozmyslowicz,T., Ostrowski,M., and Ratajczak,M.Z. (1999). Evidence that human haematopoietic stem cells (HSC) do not reside within the CD34+KIT- cell population. Ann. Transplant. *4*, 22-30.

Ratajczak,M.Z., Kucia,M., Reca,R., Majka,M., Janowska-Wieczorek,A., and Ratajczak,J. (2004). Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells 'hide out' in the bone marrow. Leukemia *18*, 29-40.

Reddel,R.R., Bryan,T.M., and Murnane,J.P. (1997). Immortalized cells with no detectable telomerase activity. A review. Biochemistry (Mosc.) *62*, 1254-1262.

Reubinoff,B.E., Pera,M.F., Fong,C.Y., Trounson,A., and Bongso,A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol *18*, 399-404.

Reya,T. and Clevers,H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843-850.

Reya,T., Morrison,S.J., Clarke,M.F., and Weissman,I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature. *414*, 105-111.

Reyes,M., Lund,T., Lenvik,T., Aguiar,D., Koodie,L., and Verfaillie,C.M. (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. Blood *98*, 2615-2625.

Richards,M., Fong,C.Y., Chan,W.K., Wong,P.C., and Bongso,A. (2002). Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat. Biotechnol. *20*, 933-936.

Robert,J.S. (2004). Model systems in stem cell biology. Bioessays *26*, 1005-1012.

Roecklein,B.A. and Torok-Storb,B. (1995). Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes. Blood *85*, 997-1005.

Rolando,N., Clapperton,M., Wade,J., Panetsos,G., Mufti,G., and Williams,R. (2000a). Granulocyte colony-stimulating factor improves function of neutrophils from patients with acute liver failure. Eur. J. Gastroenterol. Hepatol. *12*, 1135-1140.

Rolando,N., Clapperton,M., Wade,J., and Wendon,J. (2000b). Administering granulocyte colony-stimulating factor to acute liver failure patients corrects neutrophil defects. Eur. J. Gastroenterol. Hepatol. *12*, 1323-1328.

Ros,J.E., Libbrecht,L., Geuken,M., Jansen,P.L., and Roskams,T.A. (2003a). High expression of MDR1, MRP1, and MRP3 in the hepatic progenitor cell compartment and hepatocytes in severe human liver disease. J. Pathol. *200*, 553-560.

Ros,J.E., Roskams,T.A., Geuken,M., Havinga,R., Splinter,P.L., Petersen,B.E., LaRusso,N.F., van der Kolk,D.M., Kuipers,F., Faber,K.N., Muller,M., and Jansen,P.L. (2003b). ATP binding cassette transporter gene expression in rat liver progenitor cells. Gut *52*, 1060-1067.

Roskams,T., De Vos,R., and Desmet,V. (1996). 'Undifferentiated progenitor cells' in focal nodular hyperplasia of the liver. Histopathology *28*, 291-299.

Roskams,T.A., Theise,N.D., Balabaud,C., Bhagat,G., Bhathal,P.S., Bioulac-Sage,P., Brunt,E.M., Crawford,J.M., Crosby,H.A., Desmet,V., Finegold,M.J., Geller,S.A.,

Gouw,A.S., Hytiroglou,P., Knisely,A.S., Kojiro,M., Lefkowitch,J.H., Nakanuma,Y., Olynyk,J.K., Park,Y.N., Portmann,B., Saxena,R., Scheuer,P.J., Strain,A.J., Thung,S.N., Wanless,I.R., and West,A.B. (2004). Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology *39*, 1739-1745.

Roufosse,C.A., Direkze,N.C., Otto,W.R., and Wright,N.A. (2004). Circulating mesenchymal stem cells. Int. J. Biochem. Cell Biol. *36*, 585-597.

Ruzicka,K., Grskovic,B., Pavlovic,V., Qujeq,D., Karimi,A., and Mueller,M.M. (2004). Differentiation of human umbilical cord blood CD133+ stem cells towards myelo-monocytic lineage. Clin. Chim. Acta *343*, 85-92.

Saji,Y., Tamura,S., Yoshida,Y., Kiso,S., Iizuka,A.S., Matsumoto,H., Kawasaki,T., Kamada,Y., Matsuzawa,Y., and Shinomura,Y. (2004). Basic fibroblast growth factor promotes the trans-differentiation of mouse bone marrow cells into hepatic lineage cells via multiple liver-enriched transcription factors. J. Hepatol. *41*, 545-550.

Sakai,Y., Jiang,J., Kojima,N., Kinoshita,T., and Miyajima,A. (2002). Enhanced in vitro maturation of fetal mouse liver cells with oncostatin M, nicotinamide, and dimethyl sulfoxide. Cell Transplant. *11*, 435-441.

Saldanha,S.N., Andrews,L.G., and Tollefsbol,T.O. (2003a). Analysis of telomerase activity and detection of its catalytic subunit, hTERT. Anal. Biochem. *315*, 1-21.

Saldanha,S.N., Andrews,L.G., and Tollefsbol,T.O. (2003b). Assessment of telomere length and factors that contribute to its stability. Eur. J. Biochem. *270*, 389-403.

Sandhu,J.S., Petkov,P.M., Dabeva,M.D., and Shafritz,D.A. (2001). Stem cell properties and repopulation of the rat liver by fetal liver epithelial progenitor cells. Am. J. Pathol. *159*, 1323-1334.

Scharenberg,C.W., Harkey,M.A., and Torok-Storb,B. (2002). The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood *99*, 507-512.

Schatten,G., Smith,J., Navara,C., Park,J.H., and Pedersen,R. (2005). Culture of human embryonic stem cells. Nat. Methods *2*, 455-463.

Schmidt,C., Bladt,F., Goedecke,S., Brinkmann,V., Zschiesche,W., Sharpe,M., Gherardi,E., and Birchmeier,C. (1995). Scatter factor/hepatocyte growth factor is essential for liver development. Nature *373*, 699-702.

Schuldiner,M., Yanuka,O., Itskovitz-Eldor,J., Melton,D.A., and Benvenisty,N. (2000). Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A *97*, 11307-11312.

Schwartz,R.E., Reyes,M., Koodie,L., Jiang,Y., Blackstad,M., Lund,T., Lenvik,T., Johnson,S., Hu,W.S., and Verfaillie,C.M. (2002). Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J. Clin. Invest *109*, 1291-1302.

Seki,S., Kitada,T., Sakaguchi,H., Iwai,S., Kawada,N., Hayashi,Y., and Kim,S.R. (2003). Expression of progenitor cell markers in livers with fulminant massive necrosis. Hepatol. Res. *25*, 149-157.

Selden,C., Chalmers,S.A., Jones,C., Standish,R., Quaglia,A., Rolando,N., Burroughs,A.K., Rolles,K., Dhillon,A., and Hodgson,H.J. (2003). Epithelial colonies cultured from human explanted liver in subacute hepatic failure exhibit hepatocyte, biliary epithelial, and stem cell phenotypic markers. Stem Cells *21*, 624-631.

Selden,C. and Hodgson,H. (2004). Cellular therapies for liver replacement. Transpl. Immunol. *12*, 273-288.

Shamblott,M.J., Axelman,J., Wang,S., Bugg,E.M., Littlefield,J.W., Donovan,P.J., Blumenthal,P.D., Huggins,G.R., and Gearhart,J.D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc. Natl. Acad. Sci. U. S. A *95*, 13726-13731.

Sharma,A.D., Cantz,T., Richter,R., Eckert,K., Henschler,R., Wilkens,L., Jochheim-Richter,A., Arseniev,L., and Ott,M. (2005). Human cord blood stem cells generate human cytokeratin 18-negative hepatocyte-like cells in injured mouse liver. Am. J. Pathol. *167*, 555-564.

Sharma,G.G., Gupta,A., Wang,H., Scherthan,H., Dhar,S., Gandhi,V., Iliakis,G., Shay,J.W., Young,C.S., and Pandita,T.K. (2003). hTERT associates with human telomeres and enhances genomic stability and DNA repair. Oncogene *22*, 131-146.

Shen,C.N., Slack,J.M., and Tosh,D. (2000). Molecular basis of transdifferentiation of pancreas to liver. Nat. Cell Biol. *2*, 879-887.

Shimano,K., Satake,M., Okaya,A., Kitanaka,J., Kitanaka,N., Takemura,M., Sakagami,M., Terada,N., and Tsujimura,T. (2003). Hepatic oval cells have the side population phenotype defined by expression of ATP-binding cassette transporter ABCG2/BCRP1. Am. J. Pathol. *163*, 3-9.

Shiota,G., Kunisada,T., Oyama,K., Udagawa,A., Nomi,T., Tanaka,K., Tsutsumi,A., Isono,M., Nakamura,T., Hamada,H., Sakatani,T., Sell,S., Sato,K., Ito,H., and Kawasaki,H. (2000). In vivo transfer of hepatocyte growth factor gene accelerates proliferation of hepatic oval cells in a 2-acetylaminofluorene/partial hepatectomy model in rats. FEBS Lett *470*, 325-330.

Sigal,S.H., Brill,S., Fiorino,A.S., and Reid,L.M. (1992). The liver as a stem cell and lineage system. Am J Physiol *263*, G139-G148.

Simonsen,J.L., Rosada,C., Serakinci,N., Justesen,J., Stenderup,K., Rattan,S.I., Jensen,T.G., and Kassem,M. (2002). Telomerase expression extends the proliferative life-span and maintains the osteogenic potential of human bone marrow stromal cells. Nat. Biotechnol. *20*, 592-596.

Smith,L.L., Coller,H.A., and Roberts,J.M. (2003). Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. Nat. Cell Biol. *5*, 474-479.

Smith,M.A., Court EL, and Smith,J.G. (2001). Stem cell factor: laboratory and clinical aspects. Blood Rev. *15*, 191-197.

StemCell Technologies. Human Colony-Forming Cell Assays using MethoCult-Technical Manual. 2004.

Stevens,A.M., McDonnell,W.M., Mullarkey,M.E., Pang,J.M., Leisenring,W., and Nelson,J.L. (2004). Liver biopsies from human females contain male hepatocytes in the absence of transplantation. Lab Invest *84*, 1603-1609.

Storms,R.W., Trujillo,A.P., Springer,J.B., Shah,L., Colvin,O.M., Ludeman,S.M., and Smith,C. (1999). Isolation of primitive human hematopoietic progenitors on the basis of aldehyde dehydrogenase activity. Proc. Natl. Acad. Sci. U. S. A *96*, 9118-9123.

Strick-Marchand,H. and Weiss,M.C. (2002). Inducible differentiation and morphogenesis of bipotential liver cell lines from wild-type mouse embryos. Hepatology *36*, 794-804.

Strobl,H., Takimoto,M., Majdic,O., Hocker,P., and Knapp,W. (1992). Antigenic analysis of human haemopoietic progenitor cells expressing the growth factor receptor c-kit. Br. J. Haematol. *82*, 287-294.

Stuart,K.A., Riordan,S.M., Lidder,S., Crostella,L., Williams,R., and SKOUTERIS,G.G. (2000). Hepatocyte growth factor/scatter factor-induced intracellular signalling. Int. J. Exp. Pathol. *81*, 17-30.

Suda,T., Isokawa,O., Aoyagi,Y., Nomoto,M., Tsukada,K., Shimizu,T., Suzuki,Y., Naito,A., Igarashi,H., Yanagi,M., Takahashi,T., and Asakura,H. (1998). Quantitation of telomerase activity in hepatocellular carcinoma: a possible aid for a prediction of recurrent diseases in the remnant liver. Hepatology *27*, 402-406.

Suzuki,A., Iwama,A., Miyashita,H., Nakauchi,H., and Taniguchi,H. (2003). Role for growth factors and extracellular matrix in controlling differentiation of prospectively isolated hepatic stem cells. Development *130*, 2513-2524.

Suzuki,A., Zheng Yw,Y.W., Kaneko,S., Onodera,M., Fukao,K., Nakauchi,H., and Taniguchi,H. (2002). Clonal identification and characterization of self-renewing pluripotent stem cells in the developing liver. J. Cell Biol. *156*, 173-184.

Suzuki,A., Zheng,Y.W., Fukao,K., Nakauchi,H., and Taniguchi,H. (2004). Liver repopulation by c-Met-positive stem/progenitor cells isolated from the developing rat liver. Hepatogastroenterology *51*, 423-426.

Suzuki,A., Zheng,Y., Kondo,R., Kusakabe,M., Takada,Y., Fukao,K., Nakauchi,H., and Taniguchi,H. (2000a). Flow-Cytometric Separation and Enrichment of Hepatic Progenitor Cells in the Developing Mouse Liver. Hepatology *32*, 1230-1239.

Suzuki,T., Higgins,P.J., and Crawford,D.R. (2000b). Control selection for RNA quantitation. Biotechniques *29*, 332-337.

Szilvassy,S.J. (2003). The biology of hematopoietic stem cells. Arch. Med. Res. *34*, 446-460.

Szyper-Kravitz,M., Uziel,O., Shapiro,H., Radnay,J., Katz,T., Rowe,J.M., Lishner,M., and Lahav,M. (2003). Granulocyte colony-stimulating factor administration upregulates

telomerase activity in CD34+ haematopoietic cells and may prevent telomere attrition after chemotherapy. Br. J. Haematol. *120*, 329-336.

Tabei,I., Hashimoto,H., Ishiwata,I., Tokieda,Y., Tachibana,T., Akahori,M., Kyouda,S., Kubo,H., Yanaga,K., Yamazaki,Y., Takahashi,S., Sato,K., and Ishikawa,H. (2003). New approach for the establishment of an hepatocyte cell line derived from rat early embryonic stem cells. Hum. Cell *16*, 39-46.

Tanimizu,N., Nishikawa,M., SAITO,H., Tsujimura,T., and Miyajima,A. (2003). Isolation of hepatoblasts based on the expression of Dlk/Pref-1. J. Cell Sci. *116*, 1775-1786.

Terada,N., Hamazaki,T., Oka,M., Hoki,M., Mastalerz,D.M., Nakano,Y., Meyer,E.M., Morel,L., Petersen,B.E., and Scott,E.W. (2002). Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature *416*, 542-545.

Terada,R., Yamamoto,K., Hakoda,T., Shimada,N., Okano,N., Baba,N., Ninomiya,Y., Gershwin,M.E., and Shiratori,Y. (2003). Stromal cell-derived factor-1 from biliary epithelial cells recruits CXCR4-positive cells: implications for inflammatory liver diseases. Lab Invest *83*, 665-672.

Theise,N.D. (2004). Restoring balance to liver stem cell research. J. Hepatol. *41*, 673-676.

Theise,N.D., Badve,S., Saxena,R., Henegariu,O., Sell,S., Crawford,J.M., and Krause,D.S. (2000a). Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology *31*, 235-240.

Theise,N.D., Nimmakayalu,M., Gardner,R., Illei,P.B., Morgan,G., Teperman,L., Henegariu,O., and Krause,D.S. (2000b). Liver from bone marrow in humans. Hepatology *32*, 11-16.

Theise,N.D., Saxena,R., Portmann,B.C., Thung,S.N., Yee,H., Chiriboga,L., Kumar,A., and Crawford,J.M. (1999). The canals of Hering and hepatic stem cells in humans. Hepatology *30*, 1425-1433.

Theise,N.D., Yao,J.L., Harada,K., Hytiroglou,P., Portmann,B., Thung,S.N., Tsui,W., Ohta,H., and Nakanuma,Y. (2003). Hepatic 'stem cell' malignancies in adults: four cases. Histopathology *43*, 263-271.

Theocharis,S.E., Papadimitriou,L.J., Retsou,Z.P., Margeli,A.P., Ninos,S.S., and Papadimitriou,J.D. (2003). Granulocyte-colony stimulating factor administration ameliorates liver regeneration in animal model of fulminant hepatic failure and encephalopathy. Dig. Dis. Sci. *48*, 1797-1803.

Thomas,J., Liu,F., and Link,D.C. (2002). Mechanisms of mobilization of hematopoietic progenitors with granulocyte colony-stimulating factor. Curr. Opin. Hematol. *9*, 183-189.

Thomson,J.A., Itskovitz-Eldor,J., Shapiro,S.S., Waknitz,M.A., Swiergiel,J.J., Marshall,V.S., and Jones,J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

Tomiya,T., Tani,M., Yamada,S., Hayashi,S., Umeda,N., and Fujiwara,K. (1992). Serum hepatocyte growth factor levels in hepatectomized and nonhepatectomized surgical patients [see comments]. Gastroenterology *103*, 1621-1624.

Tosh,D., Shen,C.N., and Slack,J.M. (2002a). Conversion of pancreatic cells to hepatocytes. Biochem. Soc. Trans. *30*, 51-55.

Tosh,D., Shen,C.N., and Slack,J.M. (2002b). Differentiated properties of hepatocytes induced from pancreatic cells. Hepatology *36*, 534-543.

Tosh,D. and Strain,A. (2005). Liver stem cells--prospects for clinical use. J. Hepatol. *42 Suppl*, S75-S84.

Triel,C., Vestergaard,M.E., Bolund,L., Jensen,T.G., and Jensen,U.B. (2004). Side population cells in human and mouse epidermis lack stem cell characteristics. Exp. Cell Res. *295*, 79-90.

Uchida,N., Leung,F.Y., and Eaves,C.J. (2002). Liver and marrow of adult mdr-1a/1b(-/-) mice show normal generation, function, and multi-tissue trafficking of primitive hematopoietic cells. Exp. Hematol. *30*, 862-869.

Ueki,T., Fujimoto,J., Suzuki,T., Yamamoto,H., and Okamoto,E. (1997). Expression of hepatocyte growth factor and its receptor c-met proto-oncogene in hepatocellular carcinoma. Hepatology *25*, 862-866.

Ulaner,G.A., Hu,J.F., Vu,T.H., Giudice,L.C., and Hoffman,A.R. (1998). Telomerase activity in human development is regulated by human telomerase reverse transcriptase (hTERT) transcription and by alternate splicing of hTERT transcripts. Cancer Res. *58*, 4168-4172.

Vassilopoulos,G., Wang,P.R., and Russell,D.W. (2003). Transplanted bone marrow regenerates liver by cell fusion. Nature *422*, 901-904.

Venezia,T.A., Merchant,A.A., Ramos,C.A., Whitehouse,N.L., Young,A.S., Shaw,C.A., and Goodell,M.A. (2004). Molecular signatures of proliferation and quiescence in hematopoietic stem cells. PLoS. Biol. *2*, e301.

Vogel,G. (1999). Harnessing the power of stem cells. Science *283*, 1432-1434.

Wang,J., Clark,J.B., Rhee,G.S., Fair,J.H., Reid,L.M., and Gerber,D.A. (2003a). Proliferation and hepatic differentiation of adult-derived progenitor cells. Cells Tissues. Organs *173*, 193-203.

Wang,J., Hannon,G.J., and Beach,D.H. (2000). Risky immortalization by telomerase. Nature *405*, 755-756.

Wang,P.P., Wang,J.H., Yan,Z.P., Hu,M.Y., Lau,G.K., Fan,S.T., and Luk,J.M. (2004). Expression of hepatocyte-like phenotypes in bone marrow stromal cells after HGF induction. Biochem. Biophys. Res. Commun. *320*, 712-716.

Wang,X., Al Dhalimy,M., Lagasse,E., Finegold,M., and Grompe,M. (2001). Liver repopulation and correction of metabolic liver disease by transplanted adult mouse pancreatic cells. Am. J. Pathol. *158*, 571-579.

Wang,X., Foster,M., Al Dhalimy,M., Lagasse,E., Finegold,M., and Grompe,M. (2003b). The origin and liver repopulating capacity of murine oval cells. Proc. Natl. Acad. Sci. U. S. A.

Wang,X., Ge,S., McNamara,G., Hao,Q.L., Crooks,G.M., and Nolta,J.A. (2003c). Albumin-expressing hepatocyte-like cells develop in the livers of immune-deficient mice that received transplants of highly purified human hematopoietic stem cells. Blood *101*, 4201-4208.

Wang,X., Montini,E., Al Dhalimy,M., Lagasse,E., Finegold,M., and Grompe,M. (2002). Kinetics of liver repopulation after bone marrow transplantation. Am. J. Pathol. *161*, 565-574.

Wang,X., Willenbring,H., Akkari,Y., Torimaru,Y., Foster,M., Al Dhalimy,M., Lagasse,E., Finegold,M., Olson,S., and Grompe,M. (2003d). Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature *422*, 897-901.

Wang,Y., Nan,X., Li,Y., Zhang,R., Yue,W., Yan,F., and Pei,X. (2005). Induction of umbilical cord blood-derived beta2m-c-Met+ cells into hepatocyte-like cells by coculture with CFSC/HGF cells. Liver Transpl. *11*, 635-643.

Wege,H., Chui,M.S., Le,H.T., Strom,S.C., and Zern,M.A. (2003a). In vitro expansion of human hepatocytes is restricted by telomere-dependent replicative aging. Cell Transplant. *12*, 897-906.

Wege,H., Le,H.T., Chui,M.S., Liu,L., Wu,J., Giri,R., Malhi,H., Sappal,B.S., Kumaran,V., Gupta,S., and Zern,M.A. (2003b). Telomerase reconstitution immortalizes human fetal hepatocytes without disrupting their differentiation potential. Gastroenterology *124*, 432-444.

Wognum,A.W., Eaves,A.C., and Thomas,T.E. (2003). Identification and isolation of hematopoietic stem cells. Arch. Med. Res. *34*, 461-475.

Wong,J.M. and Collins,K. (2003). Telomere maintenance and disease. Lancet *362*, 983-988.

Wormstone,I.M., Tamiya,S., Marcantonio,J.M., and Reddan,J.R. (2000). Hepatocyte growth factor function and c-Met expression in human lens epithelial cells. Invest Ophthalmol. Vis. Sci. *41*, 4216-4222.

Wulf,G.G., Luo,K.L., Jackson,K.A., Brenner,M.K., and Goodell,M.A. (2003). Cells of the hepatic side population contribute to liver regeneration and can be replenished with bone marrow stem cells. Haematologica *88*, 368-378.

Xiao,J.C., Ruck,P., Adam,A., Wang,T.X., and Kaiserling,E. (2003). Small epithelial cells in human liver cirrhosis exhibit features of hepatic stem-like cells: immunohistochemical, electron microscopic and immunoelectron microscopic findings. Histopathology *42*, 141-149.

Xu,C., Inokuma,M.S., Denham,J., Golds,K., Kundu,P., Gold,J.D., and Carpenter,M.K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nat. Biotechnol. *19*, 971-974.

Yamamoto,H., Quinn,G., Asari,A., Yamanokuchi,H., Teratani,T., Terada,M., and Ochiya,T. (2003). Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application. Hepatology *37*, 983-993.

Yamamoto,Y., Teratani,T., Yamamoto,H., Quinn,G., Murata,S., Ikeda,R., Kinoshita,K., Matsubara,K., Kato,T., and Ochiya,T. (2005). Recapitulation of in vivo gene expression during hepatic differentiation from murine embryonic stem cells. Hepatology *42*, 558-567.

Yamazaki,S., Miki,K., Hasegawa,K., Sata,M., Takayama,T., and Makuuchi,M. (2003). Sera from liver failure patients and a demethylating agent stimulate transdifferentiation of murine bone marrow cells into hepatocytes in coculture with nonparenchymal liver cells. J. Hepatol. *39*, 17-23.

Yarden,Y., Kuang,W.J., Yang-Feng,T., Coussens,L., Munemitsu,S., Dull,T.J., Chen,E., Schlessinger,J., Francke,U., and Ullrich,A. (1987). Human proto-oncogene c-kit: a new cell surface receptor tyrosine kinase for an unidentified ligand. EMBO J. *6*, 3341-3351.

Yin,A.H., Miraglia,S., Zanjani,E.D., Almeida-Porada,G., Ogawa,M., Leary,A.G., Olweus,J., Kearney,J., and Buck,D.W. (1997). AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood *90*, 5002-5012.

Yin,L., Sun,M., Ilic,Z., Leffert,H.L., and Sell,S. (2002a). Derivation, characterization, and phenotypic variation of hepatic progenitor cell lines isolated from adult rats. Hepatology *35*, 315-324.

Yin,Y., Lim,Y.K., Salto-Tellez,M., Ng,S.C., Lin,C.S., and Lim,S.K. (2002b). AFP(+), ESC-derived cells engraft and differentiate into hepatocytes in vivo. Stem Cells *20*, 338-346.

Ying,Q.L., Nichols,J., Evans,E.P., and Smith,A.G. (2002). Changing potency by spontaneous fusion. Nature *416*, 545-548.

Yu,S., Zhang,J.Z., Zhao,C.L., Zhang,H.Y., and Xu,Q. (2004). Isolation and characterization of the CD133+ precursors from the ventricular zone of human fetal brain by magnetic affinity cell sorting. Biotechnol. Lett. *26*, 1131-1136.

Yui,J., Chiu,C.P., and Lansdorp,P.M. (1998). Telomerase activity in candidate stem cells from fetal liver and adult bone marrow. Blood *91*, 3255-3262.

Zhang,M., Sell,S., and Leffert,H.L. (2003a). Hepatic progenitor cell lines from allyl alcohol-treated adult rats are derived from gamma-irradiated mouse STO cells. Stem Cells *21*, 449-458.

Zhang,Y., Bai,X.F., and Huang,C.X. (2003b). Hepatic stem cells: existence and origin. World J. Gastroenterol. *9*, 201-204.

Zhao,Y., Glesne,D., and Huberman,E. (2003). A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. Proc. Natl. Acad. Sci. U. S. A *100*, 2426-2431.

Zhou,S., Schuetz,J.D., Bunting,K.D., Colapietro,A.M., Sampath,J., Morris,J.J., Lagutina,I., Grosveld,G.C., Osawa,M., Nakauchi,H., and Sorrentino,B.P. (2001). The

ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat. Med. *7*, 1028-1034.

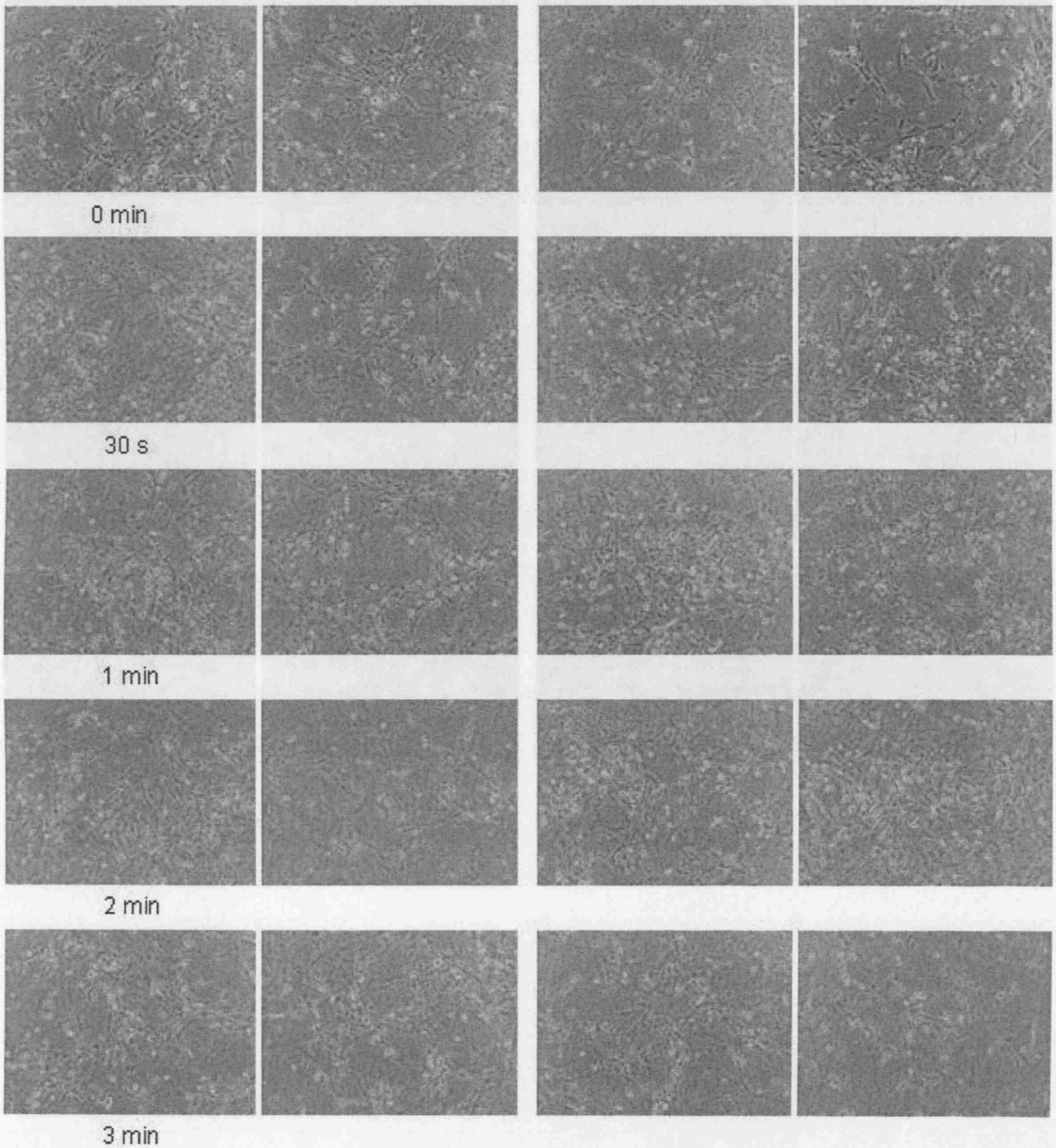
Zimmermann,S., Voss,M., Kaiser,S., Kapp,U., Waller,C.F., and Martens,U.M. (2003). Lack of telomerase activity in human mesenchymal stem cells. Leukemia *17*, 1146-1149.

Appendix 1

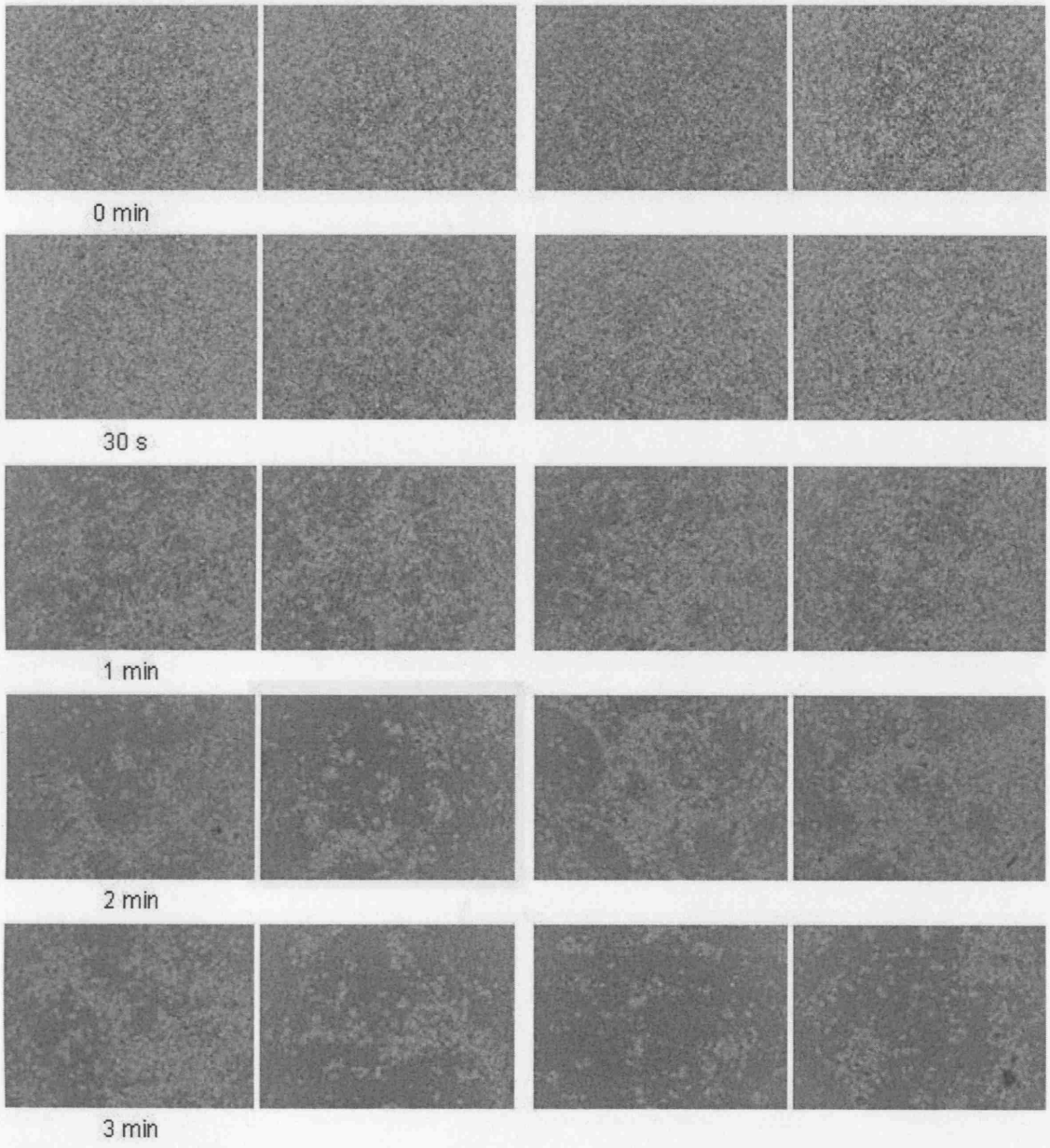
Phase contrast microscopy x100 of HS-5 feeder layers with different amounts of gamma irradiation (20Gy/min). Part (A) cells with 0min, 30s, 1min, 2min and 3min irradiation and in culture for 30 days. Part (B) cells with 0min, 1min30s and 4min irradiation and in culture for 32 days.

Part A

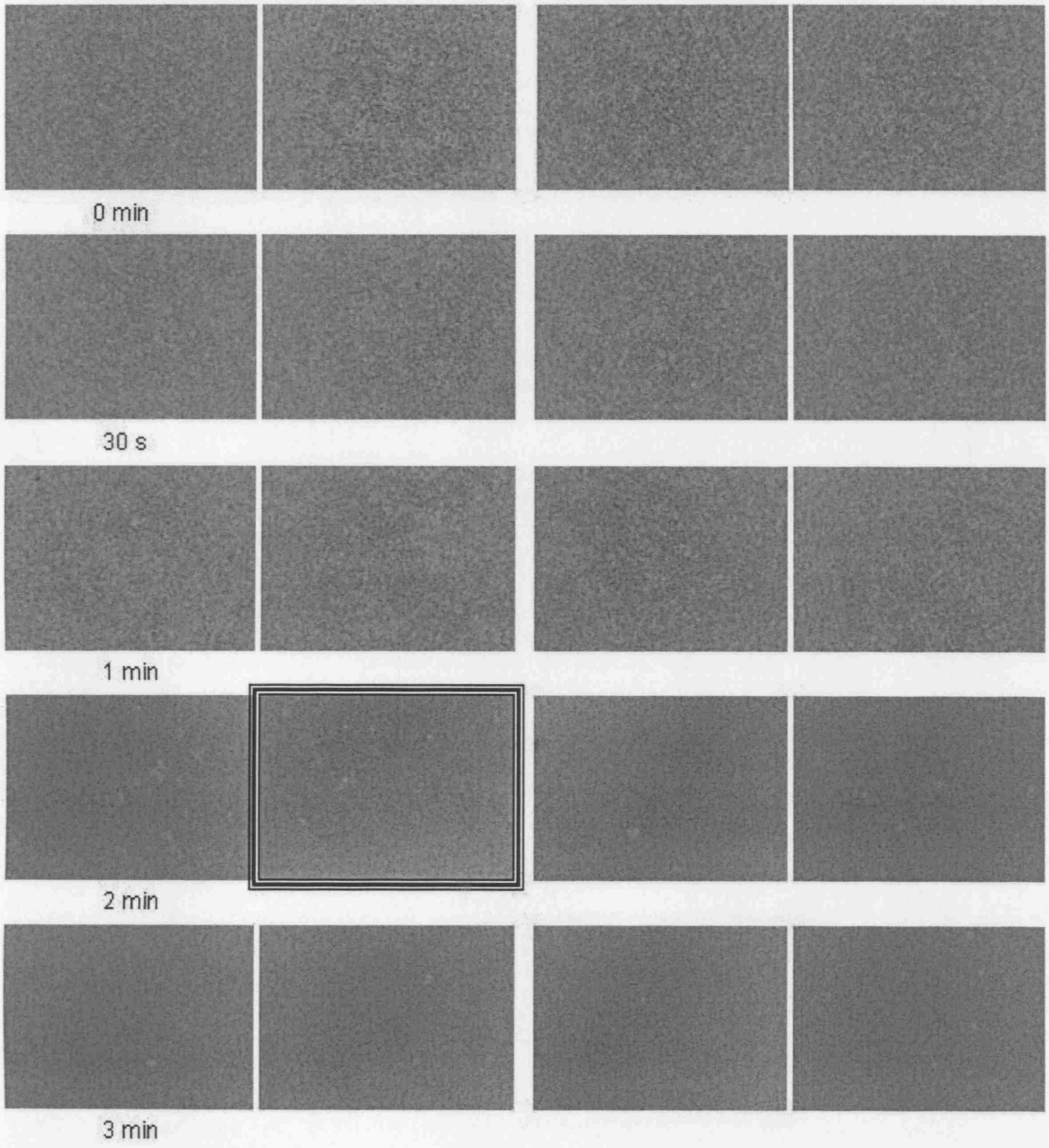
Day 1



Day 8



Day 16



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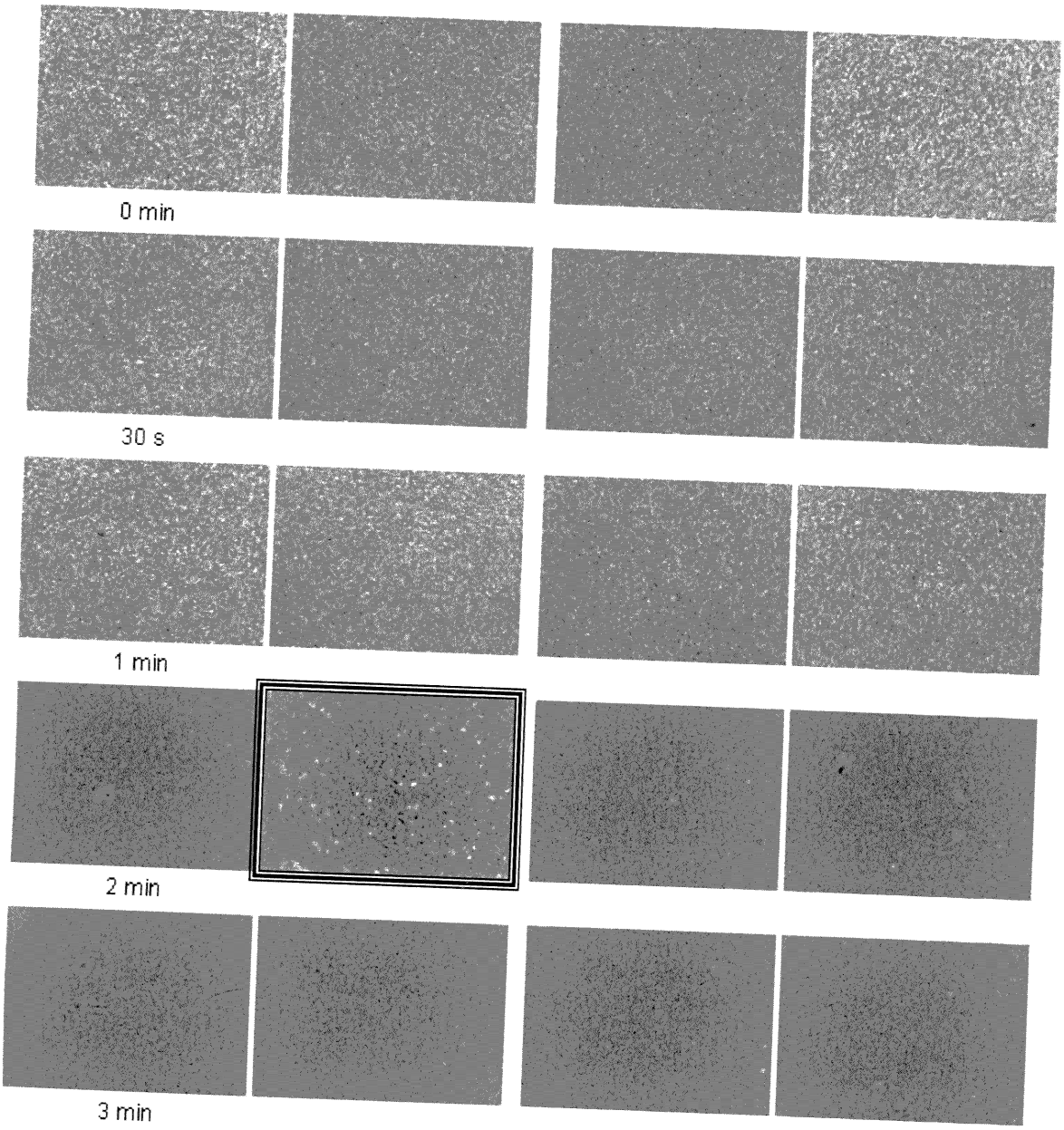
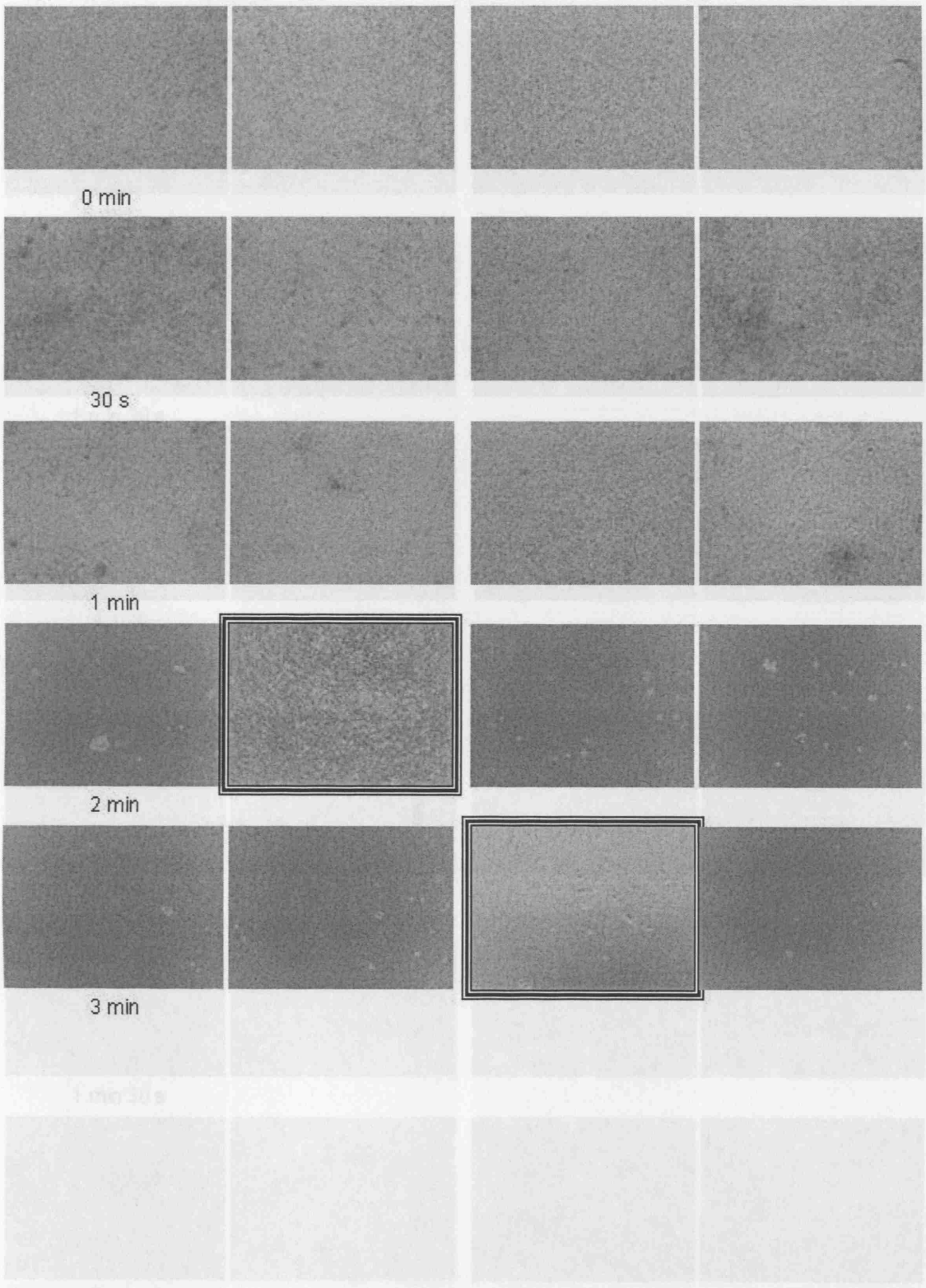
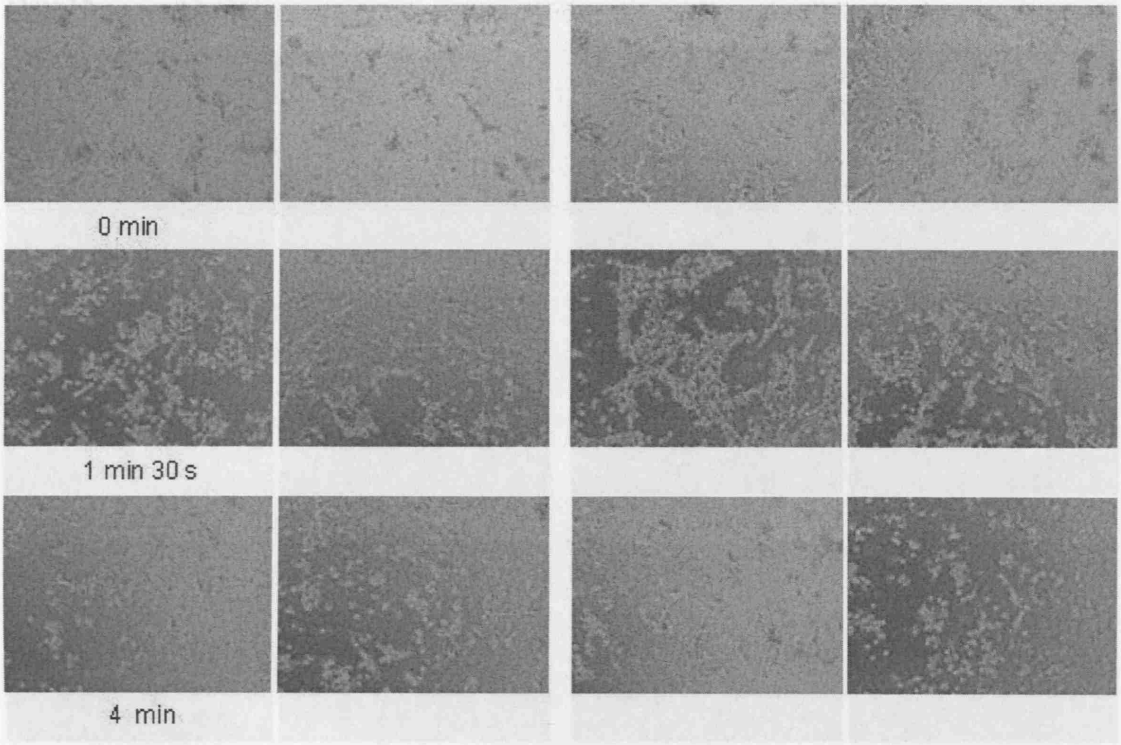


Day 30

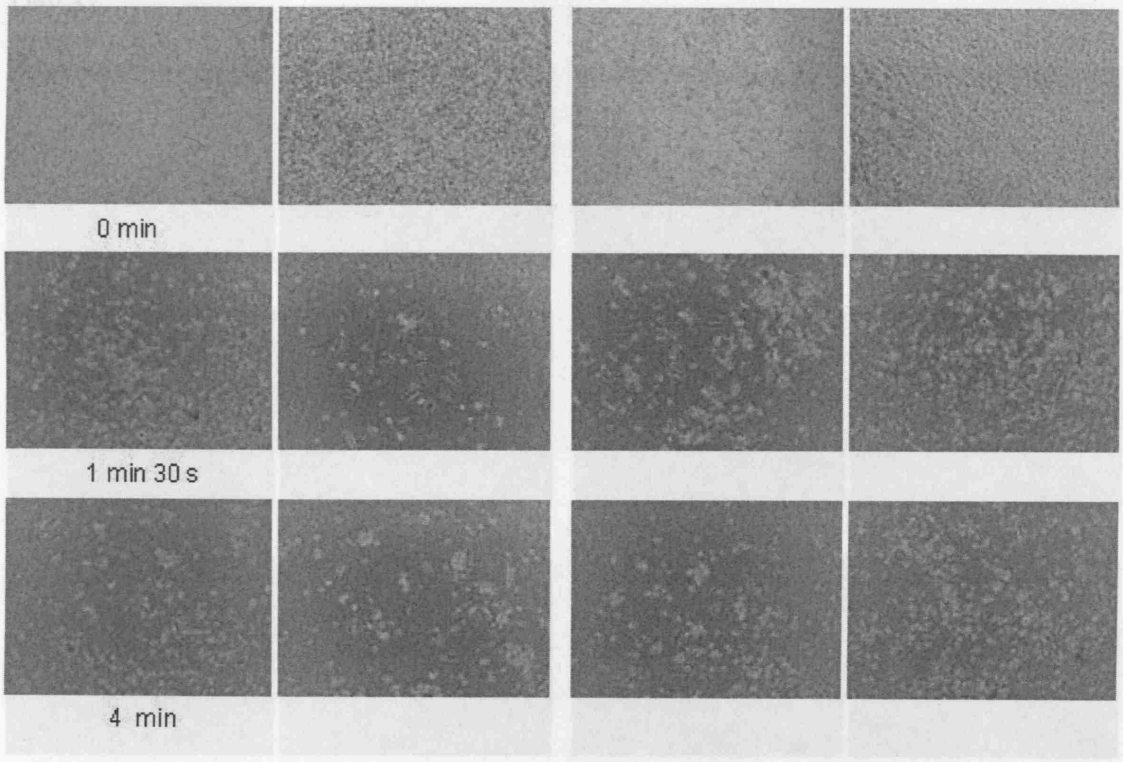


Part B

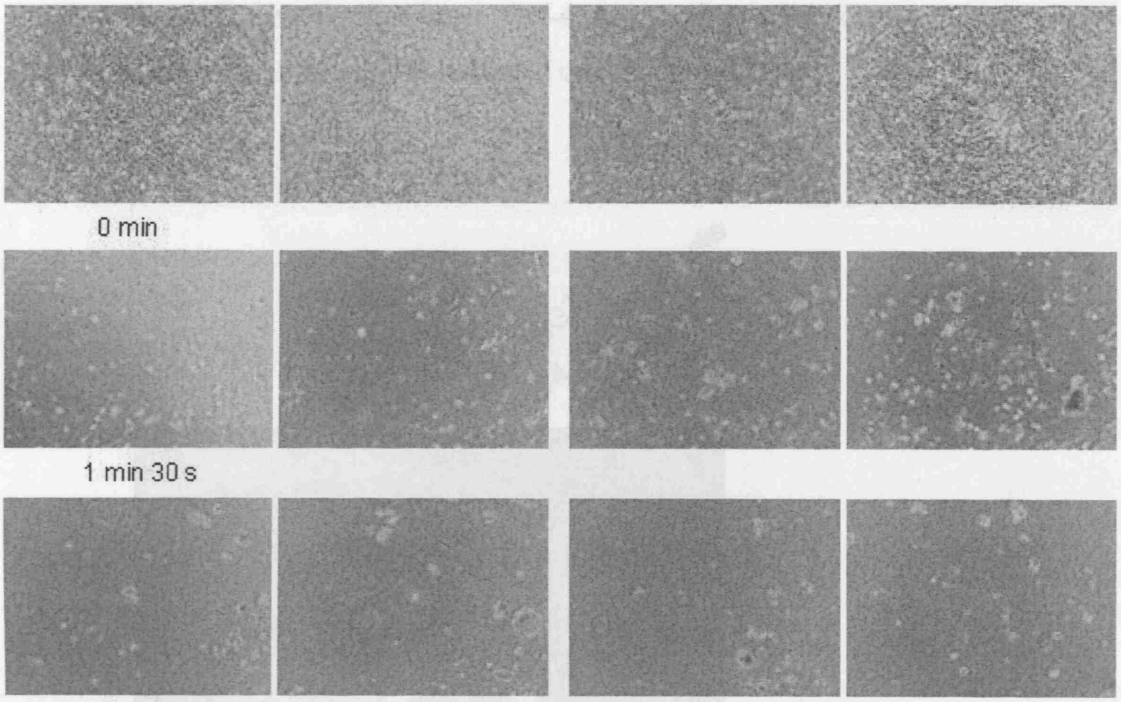
Day 1



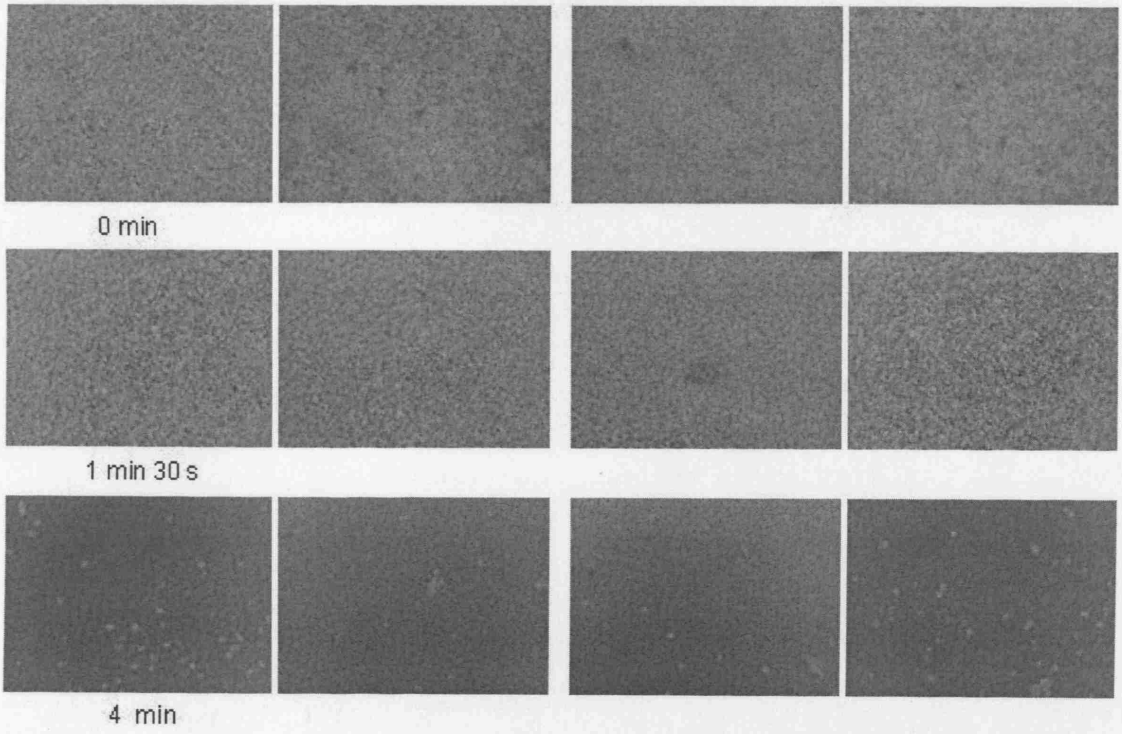
Day 6



Day 15

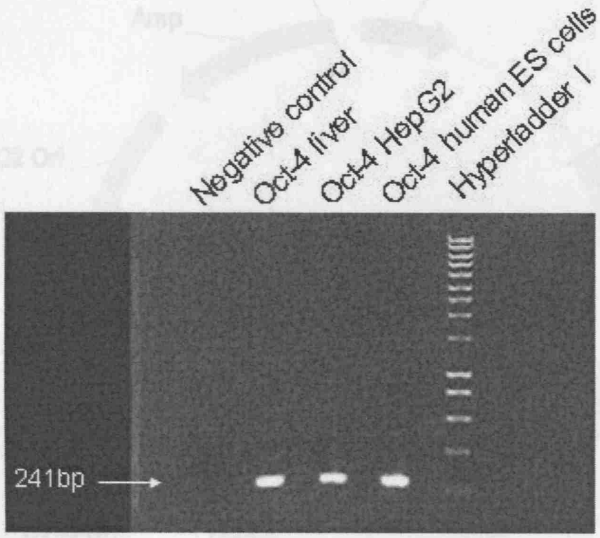


Day 32



Appendix 2

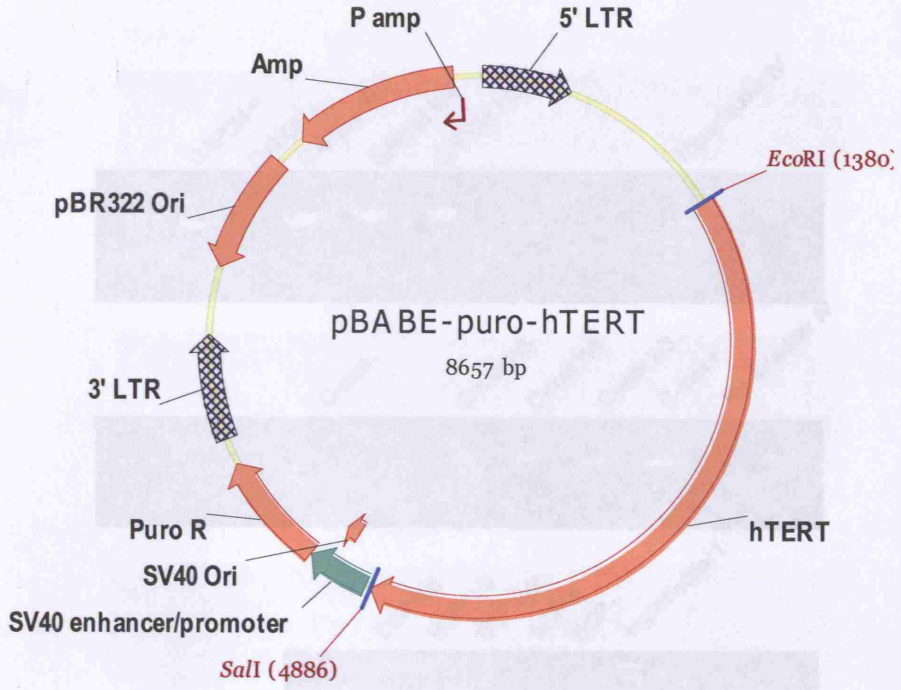
PCR gel for the marker Oct-4. Oct-4 expression was observed in human ES cells, HepG2 cells and human whole liver. Oct-4 was used as a marker in *Chapter 3* and *Chapter 4*. The size of the band is indicated on the left of the gel.



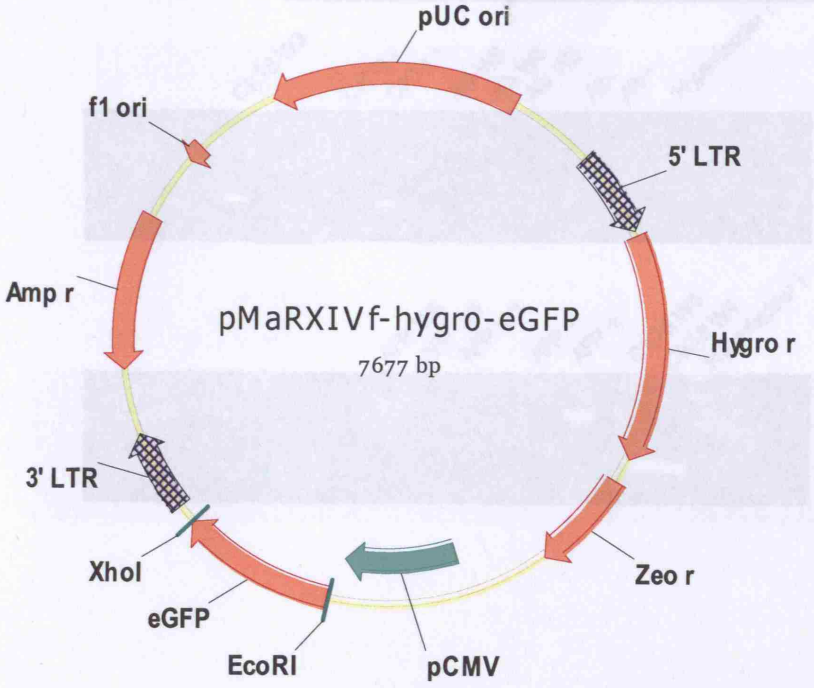
Appendix 3

Schematic vector maps of (A) pBABE-puro-hTERT (Counter et al., 1998b; Counter et al., 1998a) and (B) pMaRXIVf-hygro-eGFP (Hannon et al., 1999) constructs used in *Chapter 4*.

(A)

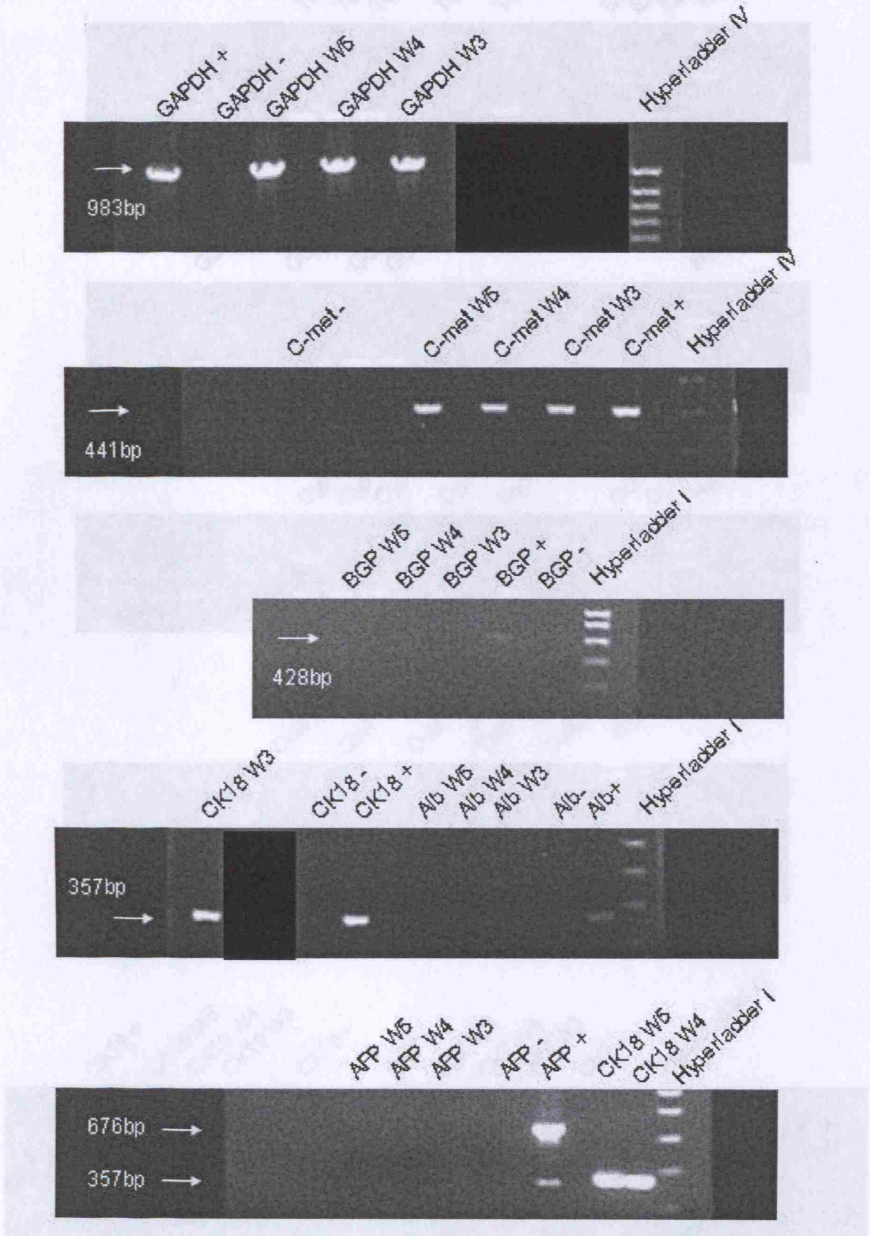


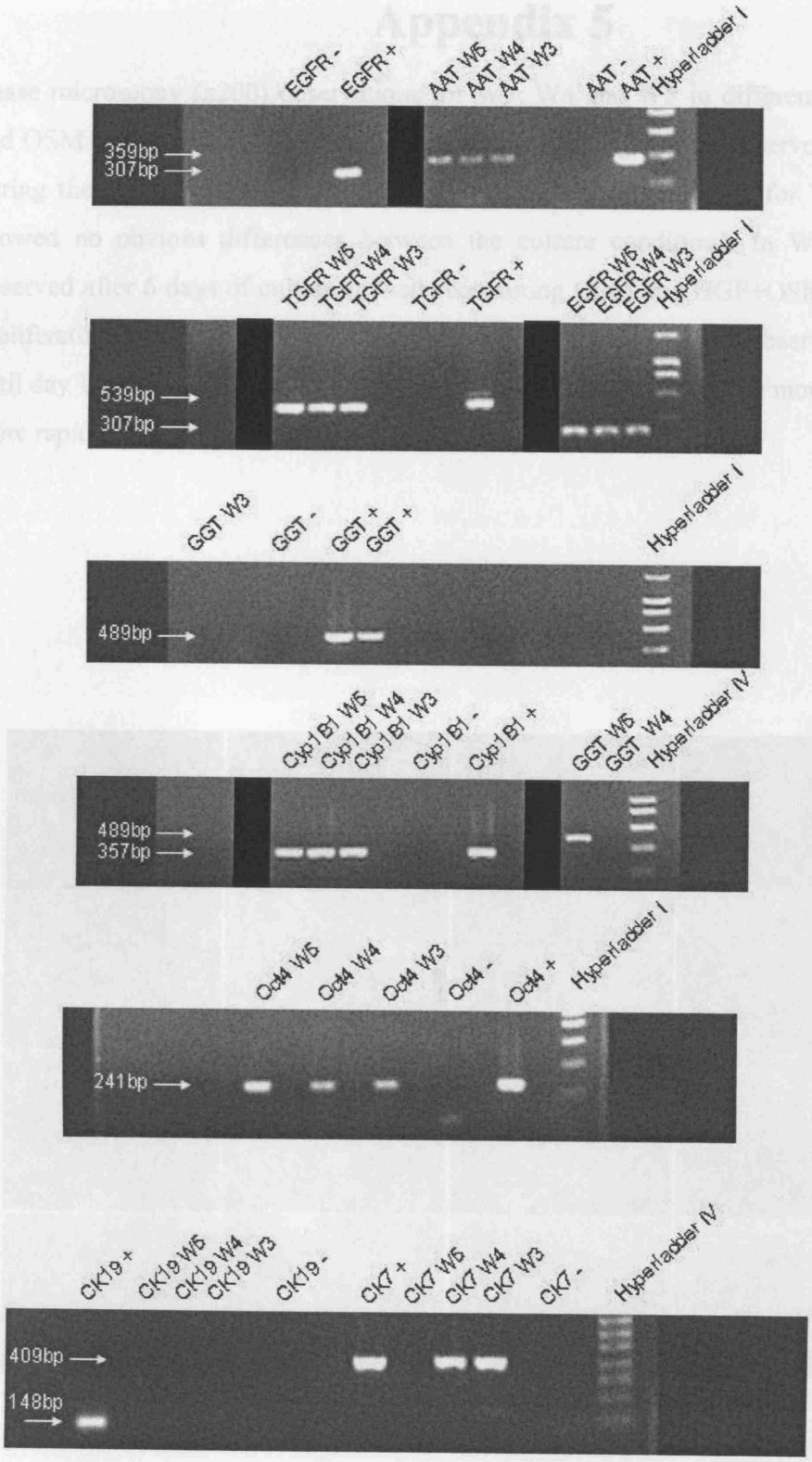
(B)



Appendix 4

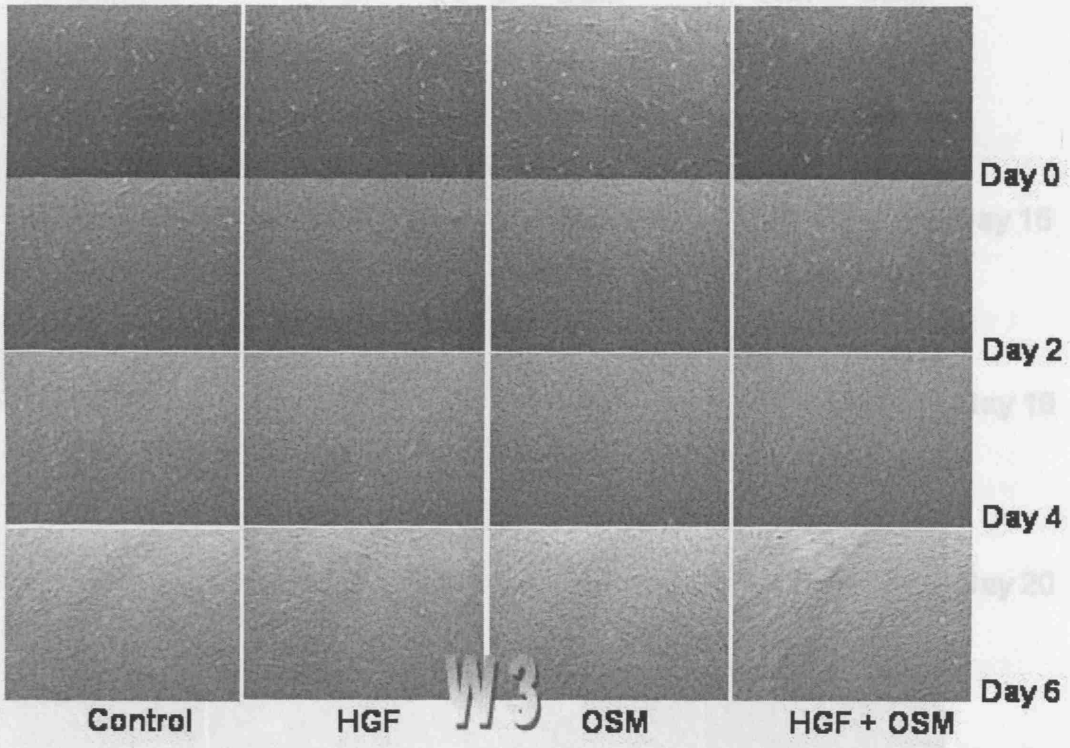
Collection of PCR gels for the different markers used to characterize the hTERT transduced colonies W3, W4 and W5 in *Chapter 4*. The size of the bands is indicated on the left of each gel. (+) positive control, (-) negative control.

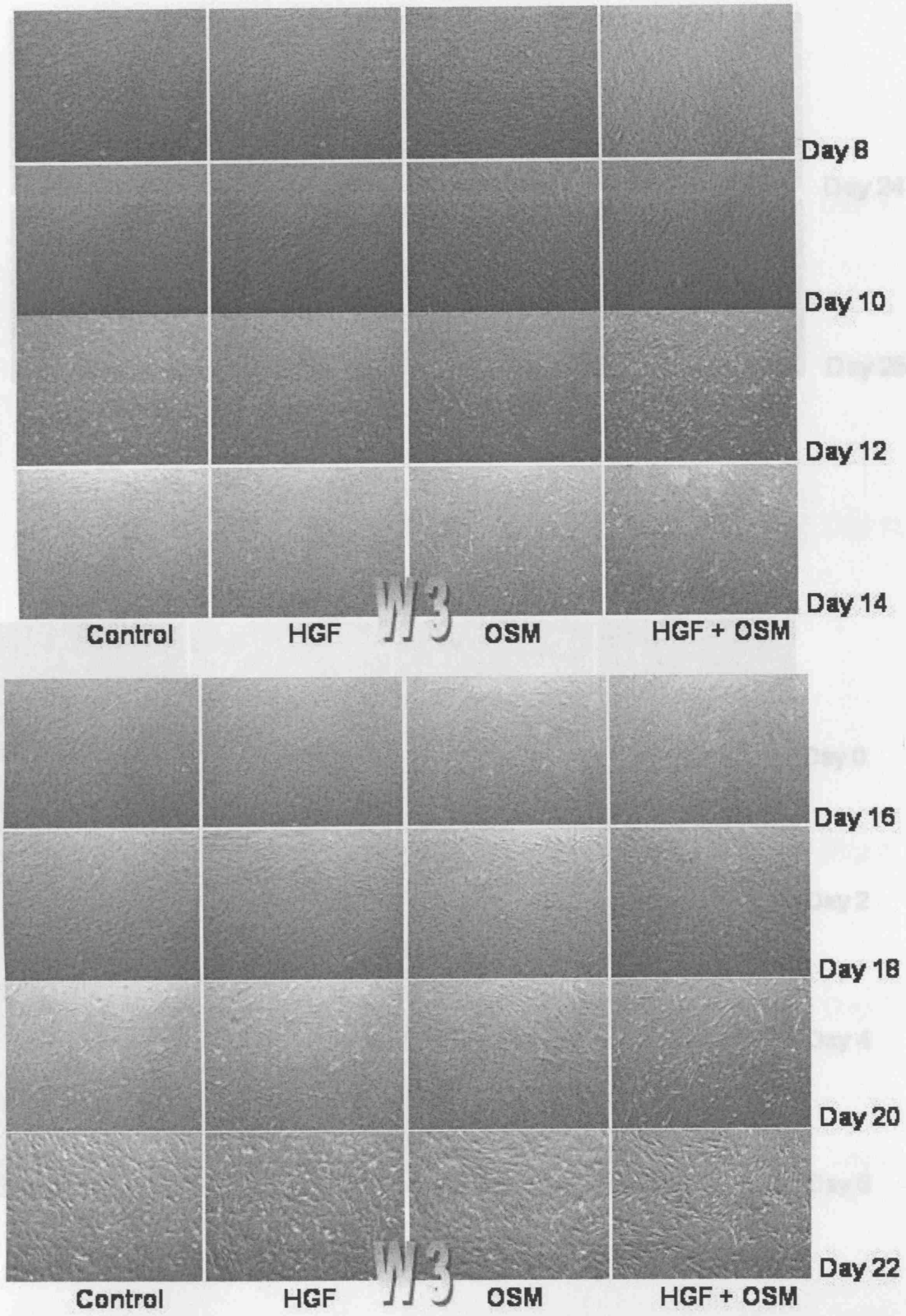


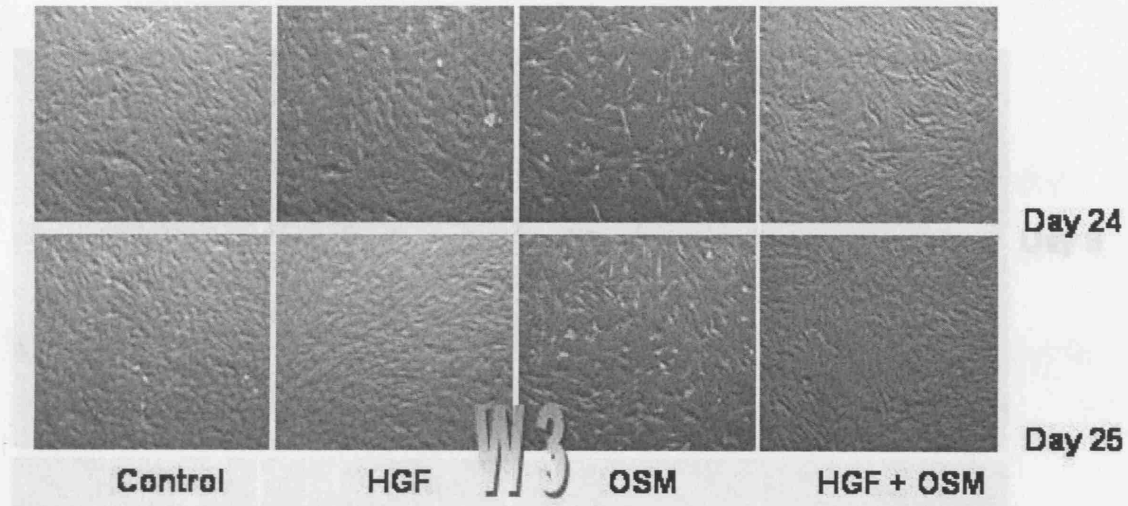

EGFR -
EGFR +
AAT W5
AAT W4
AAT W3
AAT -
AAT +
Hyperladder I
359bp
307bp
TGFR W5
TGFR W4
TGFR W3
TGFR -
TGFR +
EGFR W5
EGFR W4
EGFR W3
Hyperladder I
539bp
307bp
GGT W3
GGT -
GGT +
GGT +
Hyperladder I
489bp
Cyp1B1 W5
Cyp1B1 W4
Cyp1B1 W3
Cyp1B1 -
Cyp1B1 +
GGT W5
GGT W4
Hyperladder IV
489bp
357bp
Oct4 W5
Oct4 W4
Oct4 W3
Oct4 -
Oct4 +
Hyperladder I
241bp
CK19 +
CK19 W5
CK19 W4
CK19 W3
CK19 -
CK7 +
CK7 W5
CK7 W4
CK7 W3
CK7 -
Hyperladder IV
409bp
148bp

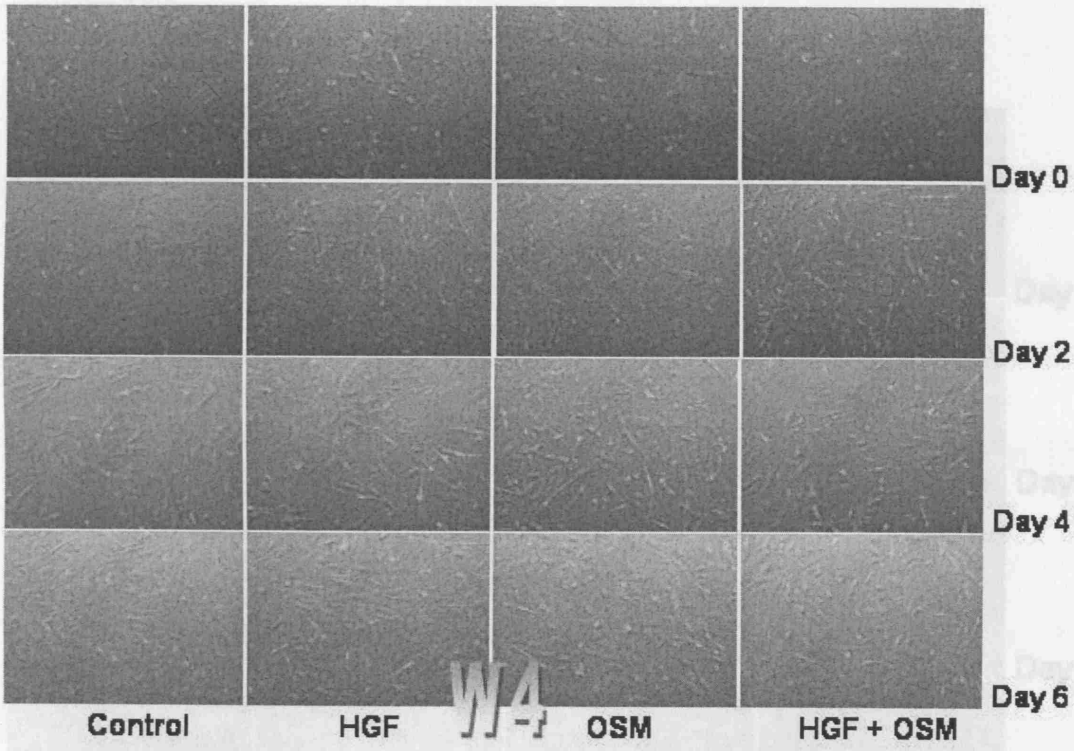
Appendix 5

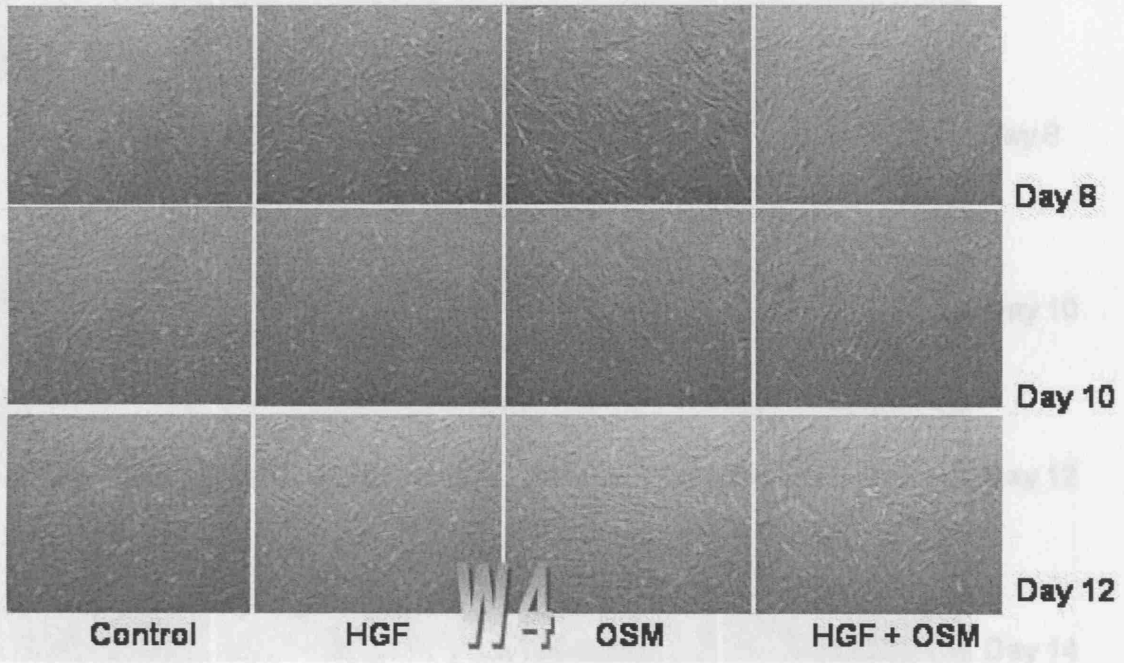
Phase microscopy (x200) observations for W3, W4 and W5 in differentiation with HGF and OSM experiment in *Chapter 4*. Morphological changes were observed every two days during the induction period, 25 days for W3 and W5 and 12 days for W4. W3 and W4 showed no obvious differences between the culture conditions. In W5, changes were observed after 6 days of culture in wells containing OSM and HGF+OSM. The cells were proliferating faster and had a more cuboidal-type appearance. These observations were seen until day 16, whereafter the cells became more fibroblastic again. The morphology was lost more rapidly in wells containing HGF+OSM.

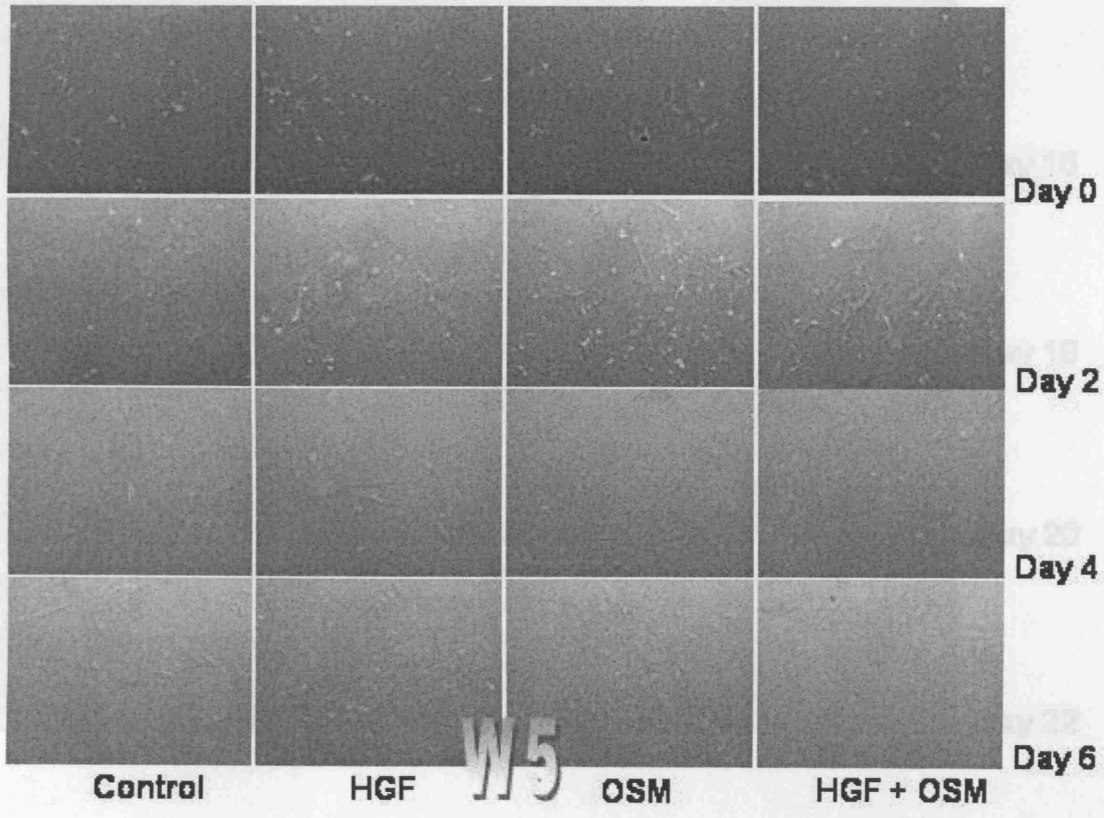


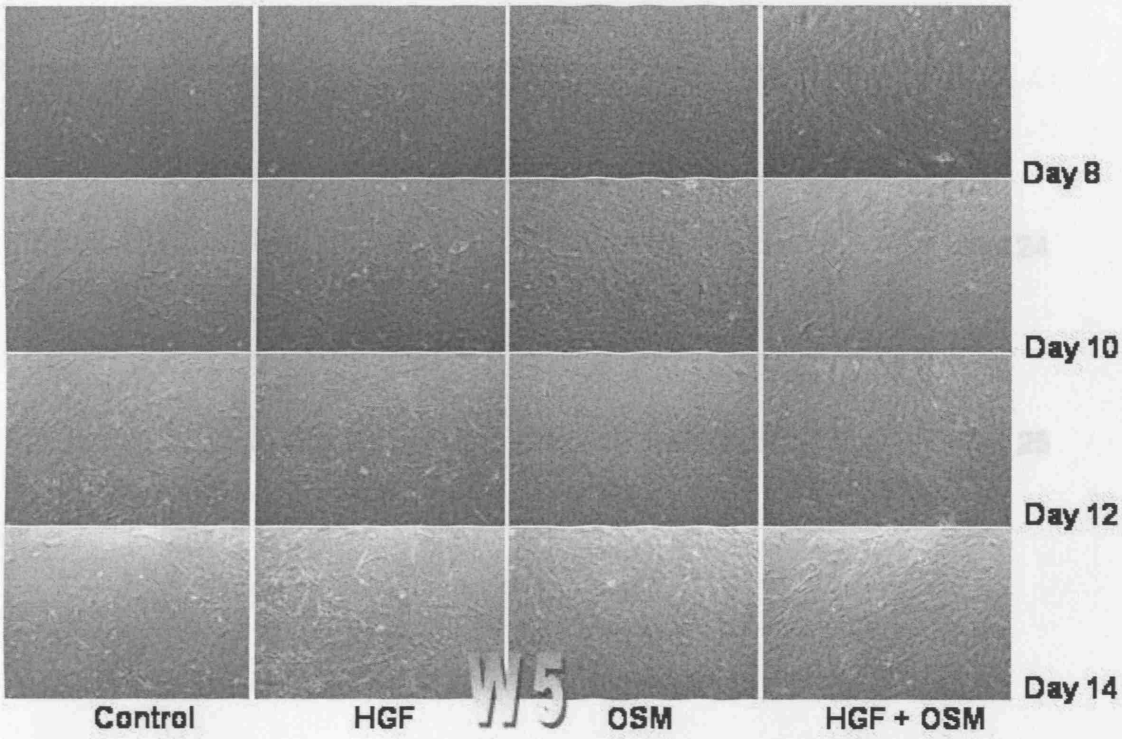

Day 8
Day 10
Day 12
Day 14
Control
HGF
OSM
HGF + OSM
Day 16
Day 18
Day 20
Day 22
Control
HGF
OSM
HGF + OSM

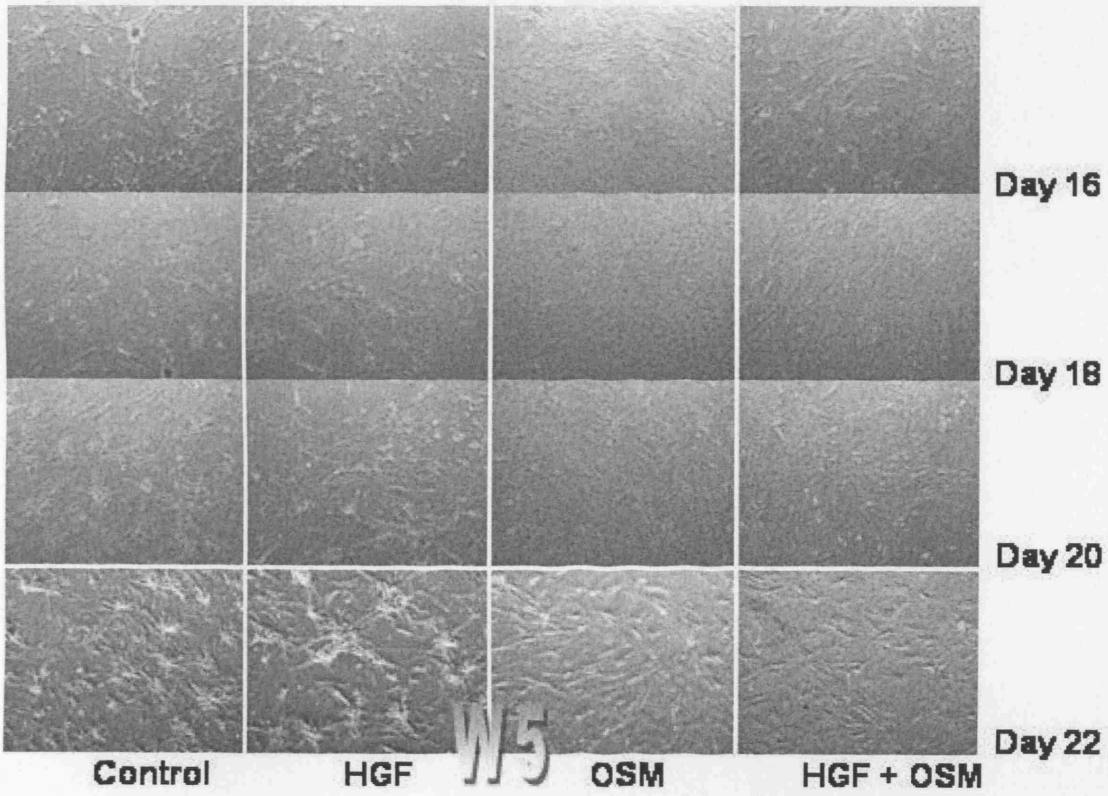

Day 24
Day 25
Control
HGF
OSM
HGF + OSM

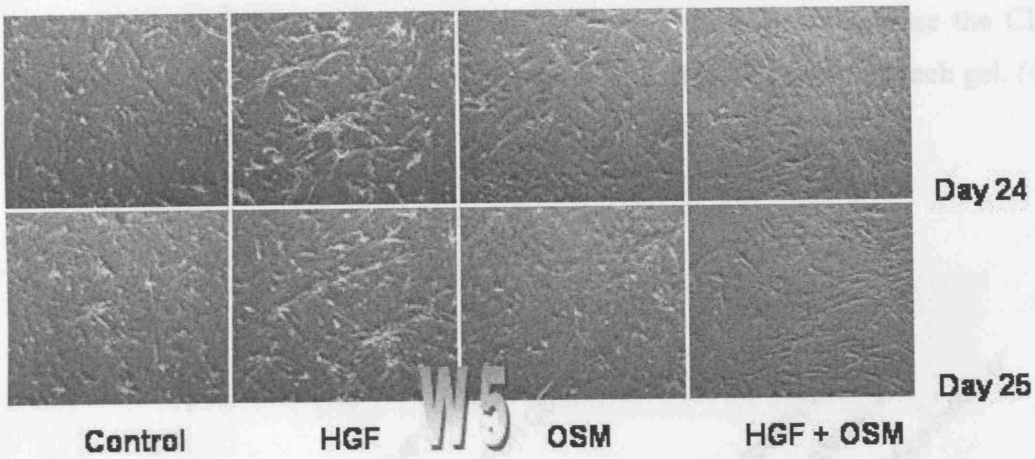

Day 0
Day 2
Day 4
Day 6
Control
HGF
OSM
HGF + OSM


Day 8
Day 10
Day 12
Control
HGF
OSM
HGF + OSM


Day 0
Day 2
Day 4
Day 6
Control
HGF
OSM
HGF + OSM

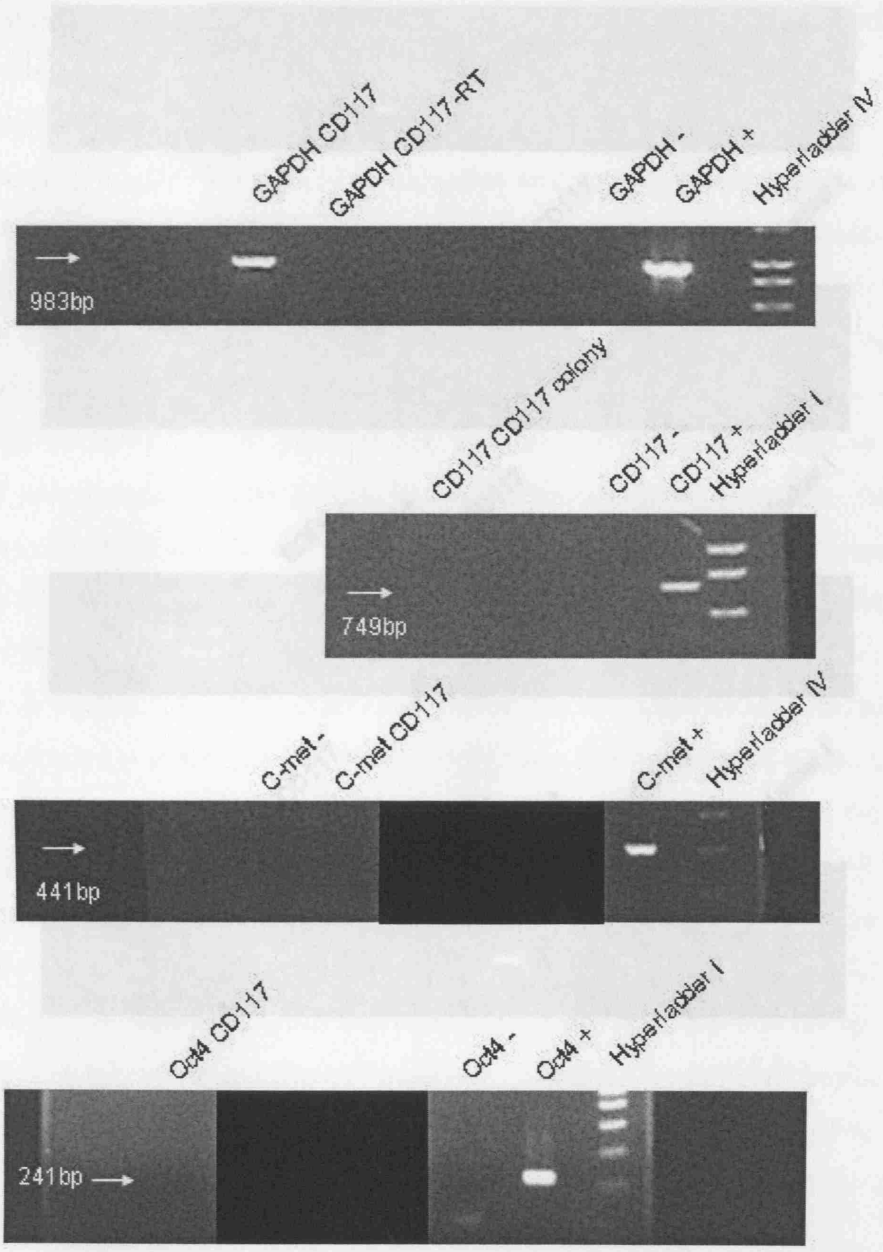

Day 8
Day 10
Day 12
Day 14
Control
HGF
OSM
HGF + OSM

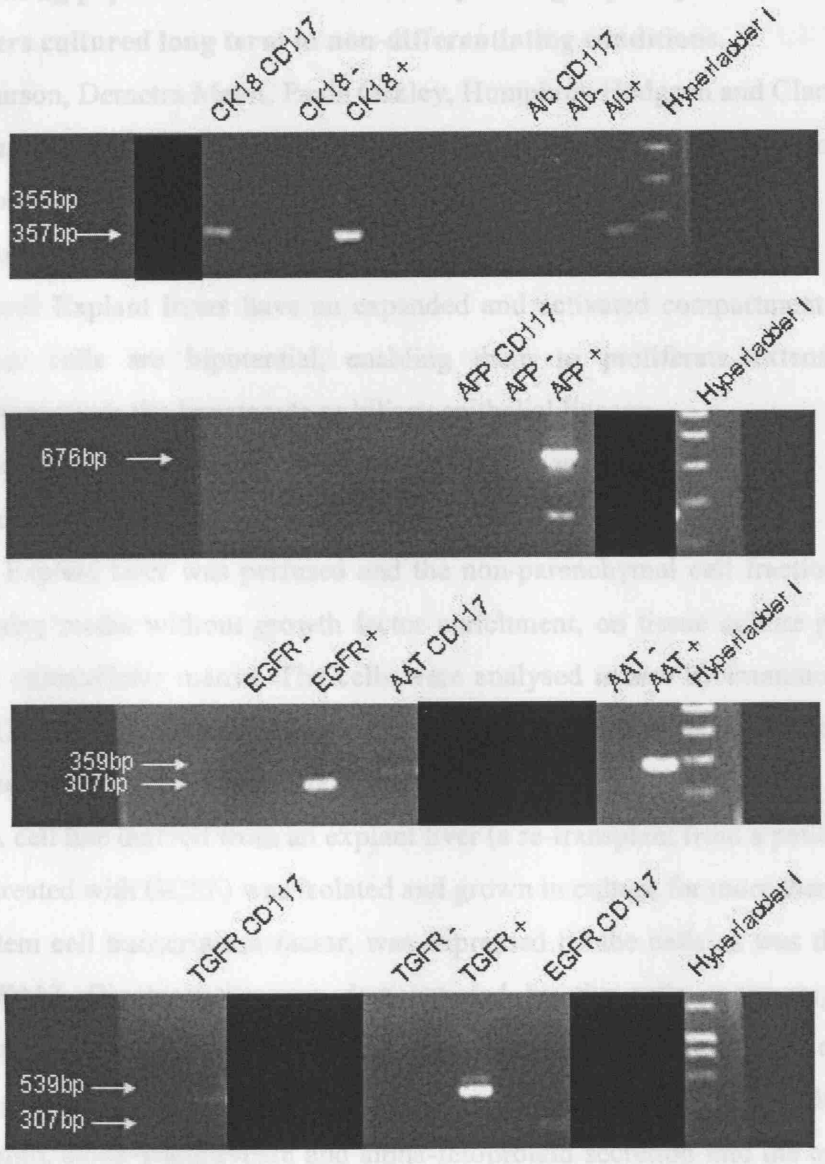

Day 16
Day 18
Day 20
Day 22
Control
HGF
OSM
HGF + OSM


Day 24
Day 25
Control
HGF
OSM
HGF + OSM

Appendix 6

Collection of PCR gels for the different markers used to characterize the $CD117^+$ cell colony in *Chapter 6*. The size of the bands is indicated on the left of each gel. (+) positive control, (-) negative control.




CK18 CD117
CK18 -
CK18 +
Alb CD117
Alb-
Alb+
Hyperladder I
355bp
357bp
AFP CD117
AFP -
AFP +
Hyperladder I
676bp
EGFR -
EGFR +
AAT CD117
AAT -
AAT +
Hyperladder I
359bp
307bp
TGFR CD117
TGFR -
TGFR +
EGFR CD117
Hyperladder I
539bp
307bp

Abstracts presented at conferences

Oral presentation – EASL Monothematic Conference, Strategies for liver support: From Stem Cells to Xenotransplantation 2003

A proliferating population of human cells expressing hepatocyte and biliary epithelial cell markers cultured long term in non-differentiating conditions

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Background: Explant livers have an expanded and activated compartment of progenitor cells. These cells are bipotential, enabling them to proliferate extensively and to differentiate towards the hepatocyte or biliary epithelial lineage.

Hypothesis: Liver progenitor cells expressing both hepatocyte and biliary epithelial cell markers can be isolated from diseased liver and kept in long term culture.

Methods: Explant liver was perfused and the non-parenchymal cell fraction isolated and cultured using media without growth factor enrichment, on tissue culture plastic without exogenous extracellular matrix. The cells were analysed *in situ* by immunocytochemistry and RT-PCR for hepatocyte, biliary epithelial and stem cell markers. Conditioned media was analysed for protein secretion by ELISA.

Results: A cell line derived from an explant liver (a re-transplant from a patient with severe rejection, treated with GCSF) was isolated and grown in culture for more than five months. Oct-4, a stem cell transcription factor, was expressed by the cells as was the cell surface marker CD117. Bipotentiality was demonstrated by the cells expressing both biliary epithelial markers, e.g. cytokeratin 19 and gamma-glutamyl transpeptidase, and hepatocyte markers e.g. Cyp1B1, cytokeratin 18 and 8. The cells were positive for the HGF-receptor c-met. Albumin, alpha-1-antitrypsin and alpha-fetoprotein secretion into the culture medium were not detectable, however, the cell population was continuously proliferating.

Conclusion: A putative bipotential progenitor cell population expressing hepatocyte, biliary epithelial and stem cell markers can be grown long term in non-differentiating conditions and can undergo continuous proliferation.

Poster – BASL Meeting 2004

Cell Colonies Expressing Both Hepatocyte and Biliary Epithelial Cell Markers can be Isolated from Non-Parenchymal Cells of Alcohol Cirrhotic Liver Explants

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Explant livers have an expanded and activated compartment of hepatocyte progenitors. Our laboratory has shown that proliferating colonies of cells expressing both hepatocyte and biliary epithelial cell markers can be isolated from sub-fulminant explants and cultured long-term. This study demonstrates how similar cells can be derived from liver with enhanced cirrhosis. **Methods:** Cells were derived from an alcohol cirrhotic liver (ACL) and a sub-fulminant liver explant by collagenase perfusion. Non-parenchymal cell fraction was cultured and colonies with a different morphological phenotype to common fibroblasts were analysed further. Colonies were transduced with a retroviral vector encoding hTERT with transduced cells selected with puromycin. Colonies were characterised using morphology, immunocytochemistry and mRNA expression by RT-PCR. **Results:** Three colonies were derived from the ACL explant and two colonies from the sub-fulminant explant. Only ACL colonies continued proliferating and survived puromycin selection. The colonies were cultured for four months after transfection before cell growth slowed down. The ACL colonies (W3, W4 and W5) each showed distinct expression patterns. All colonies expressed mRNA for hTERT, GAPDH, c-met, alpha-1-antitrypsin, CYP1B1, EGF-receptor and TGF-β-II-receptor and were negative for alpha-fetoprotein, albumin and biliary glycoprotein. W5 expressed gamma-glutamyl-transpeptidase whereas W3 and W4 did not. The protein expression patterns were different for the colonies, as seen in table below. +/- indicates both positive and negative cells present within colonies.

	Protein expression				
	c-met	CK7	CK19	CK8	CK18
W3	+	+	-	+/-	+
W4	+	+	-	+	+
W5	+	-	-	+/-	+/-

Conclusion: It is possible to isolate and culture putative progenitor cells, which exhibit both hepatocyte and biliary epithelial cell markers, from alcohol cirrhotic liver as well as sub-fulminant liver failure explants.